

When will *glasnost* reach Britain?

The British government last week defeated an attempt to reform its anachronistic Official Secrets Act, creating more problems for itself than it can hope to solve by June, as promised.

WHEN in 1911 it seemed inevitable to the British that there would be a war with Germany, word went out that British naval dockyards were thoroughly penetrated by Prussian spies and the government of the day rushed through Parliament legislation known as the Official Secrets Act, roughly half the length of this page, which has ever since been a corrosive influence in British public life — and publicly recognised as such since the report of a Royal Commission under Lord Franks (previously Sir Oliver Franks) in 1972. It has blessed (but the opposite might be the better word) Britain with a press inclined to triviality, with ministers given to irresponsibility, with an opposition more often in the dark than the interests of good government require and a voting public less well informed than it wishes (and needs) to be.

Last Friday morning, the House of Commons witnessed a brave but unsuccessful attempt to put things right. A private member — the language describes members of the House of Commons who are neither members of the government nor of the Opposition's designated list of spokesmen — who happens also to be a government supporter, failed to win the consent for a serious consideration of an amending bill. Mr Richard Shepherd's sensible but modest proposal was, in the event, defeated by the dragooned votes of the government party and the promise by the Home Secretary, Mr Douglas Hurd, that there would be a white paper in the subject in June, a debate in succeeding weeks and the government's version of an amending bill in the next parliamentary session. Even so, the government's victory was largely pyrrhic: thirty of its own supporters voted for the Shepherd bill, while its own spokesmen could not (or would not) say in what respects their own bill will be different.

Shabby

This shabby circumstance is a telling reminder both of the inherent difficulties of policies on official information and of the peculiar difficulties that arise in Britain. In most places, it is generally understood and even agreed that state secrets exist, mostly in the military field, and must be protected. It is nevertheless usually forgotten that even this concept is largely an invention of the twentieth century; at the time of the battle of Waterloo, Napoleon would have been able to estimate the forces against him by referring to official publications such as the British Army List, but would have to guess how the Duke of Wellington would dispose them. Legitimate concern about secrecy has grown with the sophistication of new weapons; in 1911, the British Navy was breaking new ground with the development of naval steam turbines, and understandably anxious that its secrets should not be used by its potential enemies. As experience of technically sophisticated weapons has grown, it has become clear that governments cannot long keep secret information about the existence of novel weapons, or of their objectives, but that deployment and performance are different matters. Thus everybody knows that the US Air Force is working on the development of aircraft that make only faint radar images, but nobody knows for certain how successfully the objective has been reached.

What else, in modern circumstances, can governments legiti-

mately claim to keep secret? Pre-planning for hypothetical conflicts has obvious importance for potential adversaries. So do the names and addresses of espionage agents (which all governments employ). Governments also have a duty to their own citizens to keep secret information about individuals with whom they have to deal on personal matters, welfare benefits or income-tax, for example. The Franks Commission also held that there is a great deal of economic information that must be safeguarded in the national interest, but probably, for reasons understandable in the early 1970s, exaggerated the case: there is, for example, no reason why the Bank of England should be required to make public the amounts it spends in any day on supporting a foreign currency, which would give fellow-players in the markets a commercial advantage, although British citizens (whose money is used) have a right to know in retrospect over suitable intervals. It would also be unwise (and often inequitable) if information about crime prevention were made public. That, innocents might think, is a sufficient definition by demonstration of the limits of legitimate government secrecy. Broadly speaking, that is the general understanding in the United States, although the Freedom of Information Act is not nearly the guarantor of open access that its admirers elsewhere pretend.

Treason

But none of this applies in Britain, thanks to the panic of 1911. Section 1 of the act, to which nobody objects, makes espionage a treasonable crime. Section 2 is a different kettle of fish altogether in that it deals with "official information" and specifies that it is a criminal offence to give or receive information of this kind unless the transfer is "authorized". Quite what constitutes official information is anybody's guess, and literally so. On a strict interpretation of the act, it would be a crime for an official to tell an enquirer the government's latest estimate of the number of AIDS cases in Britain, for example, or to explain the government's reasons for continuing to be a member of CERN, the European particle-physics laboratory at Geneva. (It would similarly be a crime for the enquirer, perhaps a journalist, to receive that information.) Exceptions arise only when the transfer of information is "authorized", which means either that the official is powerful enough to take the responsibility on his own shoulders, or that he has the consent of the minister at the head of his department.

The consequences of this state of affairs are appalling. Section 2 of the Official Secrets Act has been a licence for successive British governments' selective use of information. Officials may be "authorized" to give the good news, but not the bad news that goes with it. Or ministers may choose to leak information that puts them in a good light, confident that the validity of their assertions cannot be checked behind the cloak of official information. There are also occasions when ministers who have fallen out with their colleagues are done down by the selective leakage of information; this happened spectacularly at the end of 1986, when the government leaked a damaging letter from the Solicitor-General to the departing Secretary of State for Defence, Mr Michael Heseltine (who nevertheless supported the govern-



ment last Friday morning). One consequence is that much of the general press in Britain has been trivialized. Another is that British public life continues to perpetuate the old unfashionable distinction between the insiders and the rest. Yet, blunderbuss though it may be, the government cannot rely on the Official Secrets Act in the pursuit of reasonable objectives; it suspects, probably correctly, that juries would not convict people of criminal offences under the act when the "official information" concerned does not damage national security. It has thus been compelled to prosecute its campaign against Mr Peter Wright, the retired British spy, under the law of confidentiality rather than prosecuting all readers of his book *Spycatcher* who happen to be British under the rubric of receiving unauthorized official information.

Mr Shepherd's bill, thrown out last Friday, would not have touched the question of military security, nor even swept away Section 2 of the Official Secrets Act, but would have civilized it, not least by removing the obvious ambiguities. Under the proposed rules, prosecutions would still have been possible, but the courts would have to decide on two matters — the degree of damage done to the national interest by the disclosure of information and the extent to which the public interest is, in the process, served (which would have become a defence against a prosecution). It would also have become a defence to show that the disputed information had become public knowledge (the Peter Wright book, for example). It is a sad business that it has been thrown out, not merely because it is a lost opportunity for reform but because it shows up yet another weakness of the British system — the capacity of governments with a sufficient majority to do what they like in the House of Commons.

What happens next will be at least diverting. Plainly the House of Commons has been humiliated by the government's scorn of its rights, every bit as galling as would have been an assertion by the Reagan administration that it would not have Congress discuss the recent sale of arms to Iran until it had produced its own report on the subject. That Mr Hurd did not last week explain why the government believes Mr Shepherd's bill to have been deficient, but merely asked that the government should be given more time to make up its mind, is hardly likely to induce the conciliatory spirit the government needs in the middle of a heavy and contentious legislative programme. Can this be, people elsewhere will be asking, the place that calls itself the "mother of parliaments".

The irony is that an intelligent account of the government's difficulties could have been persuasive, giving it the chance of winning the argument as well as the vote. The British government shares with others the difficulty of drawing a distinction between questions that bear directly on the national interest and questions that are merely politically sensitive. But it is also hampered by its own constitution, and by the doctrine of collective Cabinet responsibility (the government consists solely of its ministers, who are by definition always at one) and by the convention which flows from that each minister is king in his or her department, solely responsible for its acts and policies. From this it seems to many to follow that there cannot be in Britain a Freedom of Information Act along the lines of those spreading elsewhere, for the mountains of paper passing between civil servants are in principle irrelevant to political decisions.

In fits and starts over the past eight years, the present government has shown willing to acknowledge the unreality of these conventions by administrative means, encouraging the publication of material previously withheld. But it has also seriously blotted its copybook by its petty pursuit of unauthorized leaks of information, many concerned with defence and security matters, but some in which disclosure has chiefly been politically embarrassing. What last week's defeat for the Shepherd bill implies is that it will no longer be possible to look for an administrative solution, but that the Official Secrets Act will have to be reformed. Moreover, it is difficult to think that Mr Hurd can do much better than Mr Shepherd. But he will have to try. □

One country's shame

Two US academies have chilling things to say about the imprisonment of scientists in Somalia.

GOVERNMENTS at times feel compelled to take steps that may be painful or obnoxious so as to protect some greater goal, but no country can claim a history free from acts it might now repudiate as unnecessarily harsh. But the realization that excesses will occur is no reason for condoning them, and it falls to governments where time and good fortune have provided perspective to speak out boldly when others stumble on the path.

These platitudes are prompted by the report of a delegation sponsored by the human rights committees of the National Academy of Sciences and the Institute of Medicine, which has chillingly made it clear that the government of Somalia has overstepped the bounds of acceptable behaviour in its pursuit of "national security". The delegation visited Somalia late last year, and returned with stories of torture, inhuman conditions for prisoners and a legal system that scarcely deserves that description. The academies' interest was provoked by the plight of 13 scientists, who have been imprisoned since 1982 apparently for the nonviolent expression of their beliefs or because they were perceived by the government to be political threats. The academies have been asking, since 1983, for an official explanation of the alleged crimes, but have been favoured with only one response — a letter in 1983 from the attorney general of Somalia which dealt with only six of the thirteen cases, and which stated that those concerned were charged and found guilty of "offences against national unity and security and acts of violence", adding graciously that "none of them was sentenced to death".

The recent delegation's visit to Somalia seems to have been equally frustrating. The delegation was denied permission to visit the prisoners, and met only one government official, the minister of justice and religious affairs, who had little relevant information and little sympathy for the delegation's goals. Especially maddening was the inability to meet President Mohamed Siad Barre, who appears to have controlled every aspect of life in Somalia since he came to power in the military coup of 1969. Without official help, the delegation was obliged to rely on what it was told by prisoners' wives and other relatives, as well as relief agencies working in Somalia. Their stories were horrifying — prison cells so tiny that a prisoner could neither stand straight nor lie down, brutal torture to extract bogus confessions, routine arrests without trial or charges.

What crimes may have been committed? Two of the scientists, Dr Mohamed Aden Sheikh and Mr Mohamed Yusuf Weirah, were members of the former government (respectively the ministers of health and finance) while Aden was president of the Somali Academy of Sciences and Arts at the time of his arrest. Neither has been charged or tried since his arrest in 1982, although a trial date has now been arranged for 2 February. Others arrested were alleged to be "creating a subversive organization". The charges against others are even less explicit.

Somalia is one of the poorest countries in Africa. In biblical times it was known as the Land of Punt, and was the source for frankincense and myrrh. Today, with a population somewhere around 5.5 million, it is beholden to the international financial community for survival. It has little solid ground for hope, but appears bent on snuffing out what little there may be, its best prospects for the future. Next month's trial, when the cases of three others, as well as those of Aden and Weirah, will be heard, could be an opportunity to begin to make amends. The trail will be open, according to Somali officials, and anyone who wishes to attend may do so. Although the US academies' delegation expresses little hope that the trial will be any more than a rubber stamp of the prisoner's *de facto* guilt, it does provide an opportunity for civilized countries everywhere to express their outrage and chagrin at Somalia's actions. It is an opportunity none should pass up. □

New agreement adds zest to US–Soviet exchange

- *Glasnost* rampant in the academies
- Greater access to scientific data assured

Washington

RIDING high on a wave of goodwill between their respective countries, the US National Academy of Sciences and the Academy of Sciences of the USSR on 12 January in Moscow signed a 5-year agreement that will permit a vastly expanded level of scientific cooperation between the two countries.

The new agreement calls for more individual scientific exchanges, scientific workshops and collaborative projects. It supersedes the two-year agreement between the two academies that would have expired later this year.

The signing comes on the heels of a successful meeting between members of the US and Soviet academies during last month's summit meeting between President Ronald Reagan and General Secretary Mikhail Gorbachev.

National Academy of Sciences president Frank Press says that the openness of the Soviet academy members dazzled their US counterparts. "It was almost like visiting another Western country", Press said last week after his return from Moscow. Particularly noteworthy was the availability of unexpurgated Western scientific journals, such as *Nature*.

The new agreement will be evaluated after two years to see what changes, if any, need to be made. In the initial years, exchanges of scientists will take place at the same rate as at present — up to 50 person-months a year — but Press says this will be allowed to increase if there is a greater demand. The exchanges will not be restricted to academy members, but will involve scientists chosen for accomplishments in their respective fields.

There will be up to eight workshops in the first two years of the agreement, four in each country. The proposed topics include non-linear systems in the prediction of earthquakes, vaccine development, dynamical symmetries and supersymmetries, and astrophysics. In addition, the agreement calls for cooperative research efforts on subjects ranging from condensed matter theory to nuclear reactor safety.

Press believes a significant accomplishment of the new agreement will be to permit summer workshops aimed at scientists under 30 years old. He says that the inclusion of such young people in international exchanges is unprecedented for the Soviet Union. Press is also enthusiastic about the new access US scientists

will be granted to facilities not associated with the Soviet Academy, including universities.

Although issues of human rights remain, Press says Soviet academicians have shown themselves to be effective at ameliorating violations. At a press conference in Moscow after the signing of the agreement, Soviet Academy president Guriy Marchuk said no one would be forced to stay in the Soviet Union.

It is clear that fabulous fortunes will not be made under the terms of the new agreement. A visiting Soviet scientist will receive a *per diem* allowance of \$25 for short visits, or \$500 per month for stays longer than 3 months. A US scientist visiting the Soviet Union will receive 20 roubles a day for short stays, and 400 roubles a month for longer visits.

Joseph Palca

Problems of Soviet defector

New Delhi

INDIA, Australia and the Soviet Union are involved in a diplomatic tangle over the defection of a Soviet biologist who arrived in India as a member of a tourist group. Babi Alexandra, a 25-year-old Jew, left the group during a shopping trip on 18 December to seek asylum at the Australian High Commission.

The future of the Soviet scientist remains uncertain while Australia continues diplomatic talks with the Soviet Union and Indian governments. Alexandra would have been in Australia by now but for the Indian law that a request for asylum on Indian soil from a citizen of a third country has to go through a judicial process. But once Alexandra steps out of the Australian High Commission, police will hand him over to the Soviet Embassy, which has lodged a complaint over his disappearance. Meanwhile, attempts by the Australian High Commission to get the Soviet tourist the status of a refugee have so far evoked no response from the New Delhi office of the UN High Commissioner for Refugees.

Everything points to Alexandra's being deported. In 1985, Igor Geza, from the Soviet Embassy in New Delhi, disappeared only to reappear in the United States a week later. In the 1960s, Stalin's daughter Svetlana defected to the West from New Delhi.

K.S. Jayaraman

Nuclear explosions

A NEW study by the National Resources Defense Council (NRDC) shows that there were 71 unannounced nuclear explosions at the Nevada Test Site between 1963 and 1978. NRDC says this figure provides the first clear picture of the true size of the US testing programme.

The new estimates were prepared by an examination of seismic records collected at the Seismological Laboratory at the California Institute of Technology in Pasadena by Riley Geary. NRDC figures suggest that the United States has conducted a total of 919 nuclear weapons tests since July 1945, 117 more than have been publicly announced.

J.P.

Out to get him?

HUGH DeWitt, a physicist at the Lawrence Livermore National Laboratory and an outspoken critic of US nuclear weapons-testing policy, is disputing an unfavourable performance evaluation of his work at the laboratory. DeWitt believes that the evaluation may be the harbinger of an attempt to dismiss him.

He has written to David Gardner, president of the University of California, which runs the Livermore Laboratory, to protest at the evaluation. DeWitt says the laboratory has never been happy with his criticism (see *Nature*, 329, 275; 1987), but feels that unhappiness has increased in the past year, leading to the unfavourable review.

J.P.

Research ships Finnish

FINLAND'S Rauma-Repola company last month formally handed over to the Soviet Union two deep-water research vessels, capable of operating at depths of up to 6,000 m, some 2,000 m deeper than the present limit.

The vessels, built of specially strengthened steel, were designed by a joint Finnish-Soviet team and built in secret at the Finnish yard over the past six years. Each vessel can carry a crew of three and can operate independently of the mothership for up to four and a half hours. The speed and power capacity of their batteries is said to be twice as great as those of the United States Sea Cliff and the French Nautilus vessels.

V.R.

Pin-ups banned

POSTERS and magazines portraying images of women should be banned from observatories and astronomical facilities, according to a letter published in the latest Newsletter of the American Astronomical Society. The letter, signed by 42 astronomers in the Five College Radio Astronomy Department at the University of Amherst, Massachusetts, describes such material as a form of sexual harassment whose presence "creates a hostile work environment for women".

P.C.

Expansionary Japanese budget provides little extra for science

Tokyo

THE Japanese cabinet has approved its first expansionary budget in six years. But two of the leading science-related ministries, the Ministry of International Trade and Industry (MITI) and the Science and Technology Agency, have gained little in the way of extra funds for research. Nevertheless, some new projects will be launched by diverting funds from other programmes.

The general account budget for fiscal year 1988, which, subject to Diet approval, comes into effect in April, stands at ¥56.7 million million (\$436,000 million), nearly 5 per cent up on last year. Despite a rigid policy aimed at eliminating the issuing of deficit-financing bonds by 1990, the budget has been boosted by the sale of shares of Nippon Telegraph and Telephone — when the first shares were released on the stock market last year, they shot up in price from ¥1.2 million to more than ¥3 million each and the government was able to sell the second batch at more than twice the original price. Further sales are planned this year and, provided the market can absorb them, the government stands to make millions of millions of yen. But most of the extra funds are being funnelled into public works, defence and overseas development; the increases for science and technology are miserly.

Despite their tight budgets, MITI and the agency have managed to pump sub-

stantial funds into superconductor research. The agency will get ¥3,192 million (\$25 million), including ¥2,044 million for research on the new high-temperature superconductors. The outlays include ¥895 million for a 40-tesla superconducting magnet at the National Research Institute of Metals and ¥270 million for a super high-powered electron microscope at the National Research Institute for Inorganic Materials.

MITI will get ¥3,370 million for superconductors: ¥1,060 million as 'basic technology for future industries'; ¥1,650 million to build a 70,000-kW superconducting generator from conventional superconductors under MITI's energy-saving 'Moonlight' project; and ¥140 million to assess world supplies of the rare-Earth elements needed to make the ceramics.

The Science and Technology Agency's space programme gets a substantial boost, but nearly all the extra funds go to continuing development of the H-2 rocket scheduled for launch in 1992. And the increased outlay for ocean research is almost entirely for the deep-sea submersible *Shinkai 6500* which is scheduled for completion in fiscal year 1989.

All these gains have been made at the expense of energy-related research. The agency's budget for nuclear energy has shown a steady decline over the past three years. Nevertheless ¥950 million (\$7 million) has been allocated for the a new experimental high-temperature gas-

cooled reactor at the Japan Atomic Energy Research Institute. MITI's outlays for alternative energy sources under the 'Sunshine' project have also been severely cut back. But Japan still maintains substantial alternative energy and nuclear energy research programmes.

Basic science has suddenly become a hot issue in Japan (see *Nature* 331, 6; 1988), and both MITI and the Science and Technology Agency have won substantial increases for their small basic research programmes. The agency's ERATO programme, which supports novel, sometimes eccentric, basic research will get three new projects this year on 'pico-second chemistry', 'quantum function structure' and 'information materials in plants'. The International Frontier Re-

Microchip makers gather in Texas

Washington

THE US semiconductor industry's plans to regain ground lost to its Japanese competitors took a step forward last week with the choice of Austin, Texas, as the site for a new cooperative research facility. Sematech, a consortium formed by the nation's 14 largest computer-chip manufacturers, will begin setting up soon and aims to have experimental microchip manufacturing lines running this year.

The announcement follows a congressional decision to provide Sematech with \$100 million a year for the next five years. The remainder of the consortium's \$250-million-a-year budget will come from member companies and state governments. Choice of the site was not easy: 34 states offered 135 locations. Austin came out on top because it had an excellent building, an old Data General Corporation plant, all ready to use, and could offer a close relationship with the University of Texas. The presence of the Microelectronics and Computer Technology Corporation, a similar consortium was also in Austin's favour. Around 800 people will be employed at Sematech.

Exactly what Sematech will do still remains undecided, despite a year of debate. Its emphasis is likely to be on developing production equipment that can beat the standards set by Japanese manufacturers in efficiently mass-producing sub-micron memory chips. But Japanese manufacturers appear to be so far ahead of their US competitors that some of the consortium members feel that it should concentrate on what the US already does well — designing and manufacturing custom-made chips for special purposes — rather than trying to rival Japan's skills in mass production. Others contend that individual US companies are strong enough to hold on to the lead in custom-chip design and that the industry needs to act together only in areas where Japan is supreme. A clearer focus is likely to emerge soon after a chief executive is chosen this month.

Alun Anderson

Japanese science budget

Science and Technology Agency	1986	1987	1988	(% change from 1987)
	(thousand million yen)			
Total research and development budget	427.8	432.5	440.2	(+ 1.8)
Special promotion funds	7.9	8.4	9.2	(+ 9.5)
Space	92.6	94.6	98.5	(+ 4.1)
Nuclear energy	275.2	273.4	271.5	(- 0.7)
Ocean research	6.6	7.7	9.3	(+23.4)
ERATO	2.7	3.2	3.8	(+20.8)
International frontier research system	1.1	1.5	1.5	(- 1.3)
Ministry of International Trade and Industry				
Total technology development budget	217.6	221.4	221.2	(- 0.1)
Japan key technology centre	20.5	25.0	26.0	(+ 4.0)
Basic technology for future industries	6.5	6.0	6.4	(+ 6.6)
Large-scale industrial projects	15.2	15.1	13.6	(-10.0)
Sunshine project	43.0	44.1	37.8	(-14.3)
Moonlight project	12.3	11.4	9.7	(-14.9)
Fifth-generation computer project	5.5	5.6	5.7	(+ 1.8)
Unmanned space platform	0.2	0.4	0.5	(+16.3)
Medical technology development	0.7	0.7	0.7	(0.0)
Promotion of research cooperation with developing countries	0.8	1.0	1.2	(+20.0)
Human frontiers science program	0.02	0.2	0.5*	(+135.0)

* Includes ¥300 million from the special promotion funds of the Science and Technology Agency.

search System gets a new project to study the function of the brain.

Funds for the much-discussed Human Frontier Science Program are more than doubled. But most of the money (¥300 million from the agency's 'Special Promotion Funds') will go to "more detailed studies, international meetings, and to confirm the feasibility of expected Frontiers activities". MITI officials deny that this is a continuation of the feasibility study that has been dragging on for over a year. But only ¥170 million (just over \$1 million) is assigned to actual research on biological functions. David Swinbanks

UK electronic engineers call for research-linked tax scheme

London

BRITAIN'S electronics industry is appealing to the government to reduce corporation tax and introduce a tax-incentive scheme to allow greater investment in research by private industry. Electronics manufacturers are claiming that because of an increasingly competitive world market and the need to show high levels of profit to meet City expectations, funds are being channelled into marketing existing products abroad, resulting in a rationing of research and development expenditure not before seen in this decade.

In the past, such requests for tax incentives have fallen on deaf ears. Last year an Inland Revenue and Treasury study concluded that tax-incentive schemes operating in Britain's main competitor countries produced little benefit. A government white paper (policy document) on civil research and development, published last summer (see *Nature* 328, 281; 1987), concluded that additional research and development stimulated by tax incentives in the ten countries studied in the survey was roughly one-half of the revenue forgone by the Treasury, "so that the average cost-effectiveness is low".

The Electronic Engineering Association, however, believes that the earlier study neglected to examine in sufficient detail the long-term advantages of the resulting increased investment in research. The association is reiterating the argument for tax incentives in a 20-page document submitted to the government last week advising on ways to promote industrial growth*.

The association, which represents more than 60 companies whose combined annual turnover exceeds £24,000 million, wants to see corporation tax reduced from 35 per cent to 23 per cent, with the resulting savings to the company used "at least partly" to stimulate investment in research, "rather than solely to increase dividends". The association proposes a concomitant tax allowance of 150 per cent on research and development revenue and capital expenditure, instead of the present 100 per cent. Such a move, it claims, would enable research and development investment to increase by 13 per cent each year and, on the basis of increased sales and the reduction of the imbalance in trade (currently running at a £12,000 million deficit), it would take only three or four years to compensate the government for the lost tax revenue. The association believes that it would be relatively simple for the government to ensure that tax savings were being invested in research.

The government has persistently implored industry to invest more of its

own money in research and not depend on state funds. It cites figures from the Organisation for Economic Cooperation and Development showing that less than 66 per cent of total research and development carried out by British industry in 1985 was funded from industry's own pocket, compared with 67 per cent in the United States, 72 per cent in France (in 1984), 76 per cent in Italy, 82 per cent in West Germany and 98 per cent in Japan.

The electronics companies say that of the total civil industry spending on research and development in 1985 of £5,146 million, their industry spent £1,738 million, three-quarters of which was privately funded.

The association says that electronics manufacturers are in good spirit and expect to meet targets at a level they can afford. That level, however, while acceptable to individual companies, "is not enough to stop and reverse an ever-widening trade gap in electronics products, particularly in computers and components".

Simon Hadlington

* Industrial Growth, Electronic Engineering Association, London.

Proposals to engineer change in British higher education

London

BRITAIN'S academic engineers are being asked to consider ways of restructuring engineering education to cope with rapidly changing technology and increasing financial strain. The call comes from the Engineering Council, the profession's supervisory body, in what is seen as an attempt to persuade the engineers themselves to determine their own fate, rather than have it imposed upon them during the expected restructuring of the academic science base.

The council is circulating a discussion document suggesting ways of concentrating resources. The council says changes are needed in order to solve "difficulties of a character and scale not previously encountered" by engineering education. "This situation arises from the rapidly changing technical scene, having profound implications for academic courses, against a background of human and capital resource pressures which strain the system in many ways and at many points."

In passing, the council says it does not support the suggestion of the Advisory Board for the Research Councils (ABRC), that institutions be graded depending on their mix of research and teaching.

At present, engineering education is spread between 45 universities, 35 polytechnics and central institutions and a number of institutes of higher education and colleges of technology, providing a total of 630 accredited degree courses in engineering. In 1986, 19,000 undergraduates embarked on degree courses in engineering (including about 2,000 women; the Engineering Council is campaigning to attract more women into the profession); 15,000 engineering graduates are produced annually. Additionally, around 3,800 graduates study taught masters courses, and at any one time about 5,100 students are working for research degrees across all fields of engineering.

The document takes to task academic staff for operating in a "relatively narrow

field and seldom find themselves in a team of like specialists numbering more than three or four. Often they plough a lone furrow." The council believes that small departments in which academics work in small, specialized teams "can be very inefficient or lacking in vitality".

One of the main concerns is the increasing difficulty of departments in replacing expensive equipment, to remain representative of modern industry. But the document points out that existing specialized laboratories and equipment are under-used and that "given a finite total resource there are strong arguments for locating at least major equipment installations in a smaller number of centres than exist at present".

The council proposes three 'modes' for restructuring of degree course teaching, emphasizing that an increase in student numbers remains a priority. The details of each mode have been left deliberately vague in an attempt to achieve a high degree of consensus among the council's constituents, and to avoid provoking the level of alarm that was engendered by the ABRC's document.

'Mode A' proposes a straight reduction in the number of centres offering engineering higher education, achieved by mergers or closures, with concomitant expansion of the remaining centres.

'Mode B' would retain the existing number of centres but reduce the number offering honours degree courses.

'Mode C' would develop a limited number of centres of specialism in certain fields, regardless of the actual number of centres offering engineering education.

The Engineering Council itself seems to favour Mode A; it is likely that the remaining two options, if adopted, would eventually evolve into a network of fewer but larger centres — that is, Mode A.

Simon Hadlington

* Restructuring of Engineering Higher Education. (The Engineering Council, London.)

More French researchers getting their first job only after 30

Paris

AN unpublished statistical survey of recruitment within France's largest state research employer, the Centre National de la Recherche Scientifique (CNRS), shows that the new crop of young scientists are not so young. The mean age of researchers at the lowest entry grade is now 30 years. An informal organization representing candidates for admission to CNRS, calling itself the *Collectif des Admissibles*, sees in these figures evidence of the government campaign to persuade young scientists to look outside the public sector for employment (see *Nature* 329, 380; 1987). But within the CNRS administration, the interpretation is more prosaic; the figures simply reflect a general tendency to stay longer within tertiary education before seeking employment.

Since 1984, CNRS researchers have had civil service status, giving them jobs for life once accepted into the system. In 1986, with the arrival of a new, right-wing government, the former Research Minister, Alain Devaquet, took steps to stem the growth in state research appointments enjoyed under the socialists and commissioned an inquiry to look for ways to restructure the CNRS. Although this inquiry is still incomplete, there has been a one-third reduction in the number of posts available for 1987, while both the new Minister, Jacques Valade, and the CNRS director, Serge Feneuille, are known to favour replacing initial lifetime contracts by a 'trial period' of short-term postdoctoral posts, in line with other European and US procedures. In Britain and the United States, however, postgraduates finish their doctorates three or four years earlier than their French colleagues.

Increased competition for jobs has raised the threshold for admission, and hence the age of candidates. The introduction, in 1985, of a 'three-year rule' giving initially unsuccessful candidates only two more tries will raise the average age of entry even further, as even good candidates are unlikely to get in the first time. Ironically, the aim of the three-year rule, introduced when over 600 posts were available annually (compared to 398 in 1986 and 150 for 1988), was to inject young blood into the CNRS.

However, the CNRS claims the ruling cannot be blamed for the increase in age of recruits as its effects will be visible for the first time this year, while the average age of successful candidates in most disciplines has increased steadily since 1983.

Some young scientists nevertheless view the statistics with concern. Eric Bringuier, secretary of the *Collectif des Admissibles* (which won its legal action contesting the

annulment of 250 appointments in 1986 by Devaquet — see *Nature* 327, 746; 1987), says the narrow chance of joining CNRS on the first attempt means candidates have to take up "precarious and sometimes unpaid" work while waiting. Overall, 35 per cent of candidates for the lowest two grades of post received grants before being admitted, 10 per cent were unemployed and 3 per cent were doing casual work.

The *Collectif des Admissibles* should have dissolved when its members won their fight for reinstatement last year, but, explained Hervé Chneiweiss, a biologist at the Collège de France, they are still not satisfied. A CNRS statute requires researchers to stay at their laboratory for the first 18 months after taking up their appointment — ruling out travel to foreign laboratories or conferences. The *Collectif* wants retrospective application of this rule to take account of their 12-month wait for reinstatement.

The CNRS survey shows that ageing of 'new blood' in public-sector research is more apparent for some disciplines than others. In human sciences, where 152 applications were received in 1986 for 15 posts, the mean age of new appointments at the lowest grade is now 36 years. In

contrast, so few candidates now apply for jobs in nuclear and particle physics that means have little statistical value. At the Institut National de la Santé et de la Recherche Médicale (INSERM), a more determined 'new blood' policy has so far offset the ageing seen at CNRS.

The number of candidates to apply for admission in the current rounds is down on previous years. The cuts in the number of posts available, the turmoil of last year and what is seen as an 'unfavourable' government attitude to research have, says Bringuier, frightened off potential applicants. CNRS officials say the fall in applications was "only" about 200. Meanwhile, the timetable for 1988 admissions is still upset by the 1986 setback, meaning an extra six months of financial insecurity for candidates.

Peter Coles

■ Following a government-level decision, the French direct-broadcast television satellite TDF-1, due to be put into geostationary orbit by an Ariane rocket in June this year, will now be launched in the autumn. The change of plans is thought to be related to operating difficulties with the satellite's German twin, TV-SAT, launched last year. One of TV-SAT's two solar panels has remained folded despite several attempts to release it. In a diplomatic gesture by Prime Minister Jacques Chirac, TDF-1's place on flight V-24 has been offered to India for its INSAT-1C telecommunications satellite. □

Rich rewards for medical prize-winners

London

THE £0.75 million 1988 Louis Jeantet Award for Medicine, candidates for which have to be working in Western Europe, has been split three ways. The newly appointed head of the National Institute for Medical Research on the outskirts of London, John Skehel, is cited for his research on the haemagglutinin protein of influenza virus. His share of the award will be spent on developing antiviral agents that block the interaction of this protein with its cell-surface receptor. Rolf Zinkernagel, of the University of Zurich, who shared in the discovery of 'MHC restriction', is planning to use his prize money to continue investigating how the immune system deals with

viruses, the area of research for which he is cited.

The third winner, Bert Sakmann of the Max Planck Institute for Biophysical Chemistry in Göttingen, receives his award for his studies of the ionic basis of neural excitation. These have been based, in large part, on the technique of patch clamping that he developed with Erwin Neher. He will use his award to bring the techniques of molecular biology to bear on the subject, an approach he has been pursuing with the help of Japanese collaborators.

In addition to research funds the three winners each receive a personal prize of about £30,000. The prizes will be awarded in Geneva in April. Peter Newmark



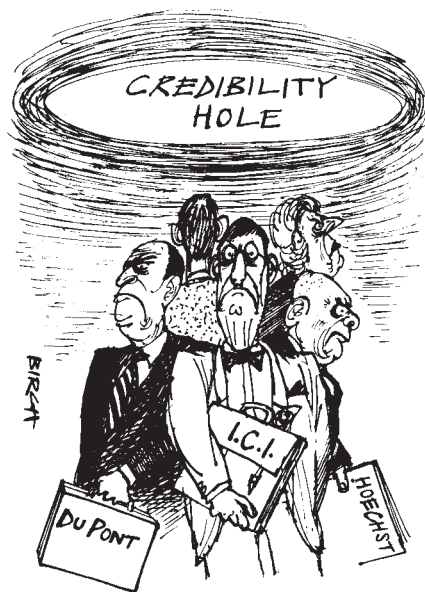
The winning Jeantet trio: Rolf Zinkernagel, John Skehel and Bert Sakmann

Depletion of ozone layer drives competitors to cooperate

Washington

INTERNATIONAL pressure for massive cuts in the production of chlorofluorocarbons (CFCs) that may damage the Earth's ozone layer is having its effect on the big chemical manufacturing companies. In the wake of the Montreal agreement, signed in September 1987, to halve the consumption of halogenated chlorofluorocarbons, thirteen competing companies from seven nations are joining together to speed the testing of alternative, less-damaging compounds.

It is the first time that so many of the world's giant chemical manufacturers — companies such as Du Pont (United States), ICI (United Kingdom), Hoechst (West Germany), and Asahi Glass (Japan) — have agreed to cooperate in an international project. They will not, however, be sharing all their trade secrets. Each company is competing to develop new alternatives to CFCs currently in use as refrigerants, polyurethane-blowing agents, solvents and propellants. But they will cooperate to test the toxicity of two agents — HFC-123 and HFC-134a — already widely canvassed as alternatives. Both agents are much less stable than existing CFCs and are much less likely to disrupt the ozone layer. Toxicity testing is



the most time-consuming job left to be done before they can be routinely used. By pooling their knowledge and by sharing expenses, the thirteen companies hope that the job can be completed as quickly as possible. Even so, a final toxicity report is not expected for 5–7 years.

Alun Anderson

Early Arctic warming may foil Antarctic expedition

London

AN expedition to the Arctic region has been set up which may shed light on the cause of the ozone destruction in the Antarctic.

A group from West Germany, France and the United States is embarking on a series of atmospheric experiments at Kiruna in northern Sweden. According to Professor Paul Crutzen of the Max Planck Institute for Chemistry, Mainz, the aim of the experiments is to see what is happening to ozone and other constituents such as ClO , NO_2 , NO and OCIO in the Arctic stratosphere to compare with the results of similar measurements made at the Antarctic over the past couple of years when investigating the 'ozone hole'. Ground-based measurements and balloon and aircraft measurements are planned and the aircraft (supplied by NASA) is equipped with a lidar to give information on the particle composition of polar stratospheric cloud. (Polar stratospheric cloud is thought to play a part in the Antarctic springtime ozone depletion.)

Some scientists have been at Kiruna since before Christmas but one problem

facing them is that the winter stratospheric 'sudden warming' is the earliest that has ever been detected.

The stratospheric warming usually does not start before mid-December and the subsequent breakdown of the stratospheric wintertime circulation does not start before the beginning of January. However, this year the warming began at the end of November (about three weeks earlier than usual). This caused an early breakdown of the Arctic polar vortex (which is both looser, warmer and more mobile than the Antarctic vortex).

Fortunately, the cold polar vortex has had time to re-establish itself although until recently the low temperatures associated with the vortex were over the North Pole. But on 18 January temperatures as low as -70°C at the 50-mbar level were reported over Kiruna. Temperatures at least as low as this, but preferably lower, are needed says Professor Karin Labitzke of Freie University, Berlin. Otherwise, the experimenters will not be able to make the measurements with the Arctic polar vortex that they have planned.

Philippa Lloyd

Remote Soviet observatory is observed

London

THE 'mysterious complex' on Mount Sanglok in Tadjikistan, which the Western media have claimed is a military installation, is simply an astrophysical observatory, *Pravda* has announced. But instead of shrugging off the allegations, *Pravda* has published a long article on the work of the observatory, including four photographs.

The Mount Sanglok facility, according to *Pravda's* Tadjikistan correspondent, A. Pokrovskii, is designed for the optical observation of celestial bodies. Although it has only a 1-m telescope, the observatory staff explained that the Tadjik 'astroclimate' — the transparency of the atmosphere and the absence of background illumination — gives almost unparalleled viewing capabilities (some 1,300–1,600 night observation hours a year) and results comparable to those obtained by much larger instruments.

The Sanglok observatory, among other things, monitors artificial Earth satellites and space-probes. Pokrovskii emphasized its observations of the lunar probes Zond-8 and Luna-16 and 17, indicating that the observatory has been operational since the late 1960s. But according to the published photographs, construction work at the site is still not completed — a powerful indication of the non-military nature of the establishment.

Western suspicions about Sanglok were aroused, Pokrovskii speculates, by satellite photographs from 800 km of this complex in the remote mountains of Tadjikistan, near the Afghanistan frontier, and linked to the Nurek hydroelectric station some 16 km away. Political 'clouds', he suggests, led Western commentators to assume that it was a laser installation.

Nevertheless, some questions about Sanglok are unanswered. Pokrovskii reports seeing a number of domes, similar to the observatory's telescope dome nearby, explained only by "another organization has an observatory there". Lasers, too, come into the picture. The tracking of modern geostationary and high-ellipticity satellites, Pokrovskii was told, requires new passive opto-electronic tracking systems. The Soviet Union is therefore developing a system to be installed at Sanglok, which is said to be "roughly similar" to the US Geodes system. There has been some discussion in the West as to whether Geodes conforms to the requirements of the Anti-Ballistic Missile treaty. But, according to Pokrovskii, the experts of both sides see no violation of the treaty from such a system.

Vera Rich

Unauthorized environmental release cleared by NIH

Washington

IN a report issued last week, the US National Institutes of Health (NIH) concludes that Gary Strobel, the Montana State University researcher who last summer conducted an unauthorized field experiment using genetically altered bacteria, did not violate any rules governing experimentation with recombinant DNA. A ten-member committee of NIH scientists, formed by director James D. Wyngaarden to investigate the incident, decided that the experiment did not involve recombinant DNA, and therefore did not fall under NIH's voluntary rules.

Strobel injected 14 elm trees with a strain of *Pseudomonas syringae* that produced an anti-mycotic agent, to see if the bacteria would protect the trees against Dutch Elm disease. To trace the movement of the bacteria, Strobel combined the *P. syringae* with a strain of *Escherichia coli* that contained a recombinant plasmid carrying a transposon coding for kanamycin resistance. Because the *E. coli* transferred the gene for kanamycin resistance to the *P. syringae* by normal bacterial conjugation, and because no other part of the recombinant plasmid remained in the *P. syringae* after integration of the transposon, the NIH committee reasoned that according to its rules the bacteria did

not contain recombinant DNA.

The committee agreed that the experiment did constitute a deliberate release of genetically altered bacteria into the environment, but this does not fall under NIH jurisdiction. Strobel received sanctions from the US Environmental Protection Agency for the release, and must find a 'responsible party' to co-sponsor any further research he undertakes. The NIH committee recommended that the definition of recombinant DNA be clarified.

The Strobel incident caused a flurry of activity in Washington, and prompted an assessment by the General Accounting Office (GAO) of the effectiveness of the NIH's institutional biosafety committees. The GAO concluded in a report, also released last week, that the majority of biosafety committees have complied with NIH guidelines, but that they contain too many members with backgrounds in genetic engineering and too few specialists in physical containment, epidemiology, ecology and large-scale fermentation techniques. Committee chairmen also confessed to ignorance of the rules governing biotechnology in the GAO study; 75 per cent had not reviewed the Coordinated Framework for the Regulation of Biotechnology, published in June of 1986.

Carol Ezzell

Initial sales of Genentech's TPA set new records

Washington

THE clot-dissolving drug TPA (tissue plasminogen activator) is well on the way to becoming the \$1,000 million money-earner that its manufacturer, Genentech, had hoped for. At the Hambrecht & Quist health care conference in California last week, Genentech announced that sales of TPA, trade-named Activase, had reached \$58 million in the last six weeks of 1987, following the drug's approval in the second week of November.

But now that public clamour to approve the drug has subsided, some critics are asking if the drug's price, ten times that of streptokinase, is really justified. Genentech's chief executive officer, Robert Swanson, lauded the sales figures, saying TPA was "the most successful new drug ever launched in the United States".

Huge publicity and high expectations have surrounded TPA from the beginning. Swanson assembled a large marketing and sales team early in the drug approval process, and representatives armed with lap-top computers were in the field, extolling the virtues of TPA to physicians

even as the US Food and Drug Administration debated potential side effects.

TPA was a magic name on Wall Street last year; the prices of Genentech and other biotechnology stocks oscillated according to rumours of its impending approval. Even hospitals have tried to cash in by advertising that they use TPA.

Although skeptics are hard to find, some worry that, because Genentech has convinced top cardiologists (many of whom have stock in the company) of the drug's superiority, doctors from rural areas and non-specialists without time to scrutinize the literature will prescribe TPA whether or not it is the best treatment for their patients.

Despite the wave of support that carried TPA into 1988, sales are likely to taper off as users acquire inventories of the drug. Stuart Weisbrod, an analyst with Prudential-Bache, attributes 75 per cent of the initial sales to "filling of the Activase pipeline", and warns that investors should not expect sales to continue at the present level. He expects 1988 TPA sales to be some \$160 million.

Carol Ezzell

Vaccine project is scrapped

Sydney

THE problems of academic scientists collaborating with industry are underlined by events at the Division of Animal Health of the Commonwealth Scientific and Industrial Organization (CSIRO).

For a number of years, CSIRO scientists have been trying to create a vaccine against two sheep parasites, *Trichostrongylus columbriformis* and *Haemonchus contortus*, otherwise known as barber's pole worm. The incident has its roots in 1984 when the Division of Animal Health received a grant for this research from the Department of Industry, Trade and Commerce. The grant came with the condition that a commercial partner must be involved, so the company Biotechnology Australia was given a share in the grant and given exclusive rights to any vaccine developed against either of the parasites at the CSIRO — even though the company made no financial contribution to the work.

Things started to go awry when Dr Edward Munn, of the Institute of Animal Physiology and Genetics Research at Babraham Hall, Cambridge, England, visited the Division of Animal Health's McMaster Laboratory last year to give a seminar on his work on antigens and in particular one from the barber's pole worm which might eventually form the basis of a vaccine.

CSIRO scientists Dr Barry Wagland and Dr Warwick Grant were sufficiently impressed to want to try Munn's antigen made from Australian worms in Australian sheep. According to the then chief of division, Dr John Dineen, he approached Biotechnology Australia but the company said it was not interested in the work.

Dineen — since retired — gave the go-ahead for a purely in-house evaluation of Munn's antigen with the stipulation that if the work were to go any further it would mean negotiating with and receiving approval from Biotechnology Australia.

In October last year, Biotechnology Australia became aware that experiments and discussions with Munn were still going on; claiming that it was prejudicial to its commercial interests, Biotechnology Australia demanded that the work be stopped and that all the experimental results be destroyed.

Had Munn's antigen eventually led to a vaccine, the intellectual property rights would have been contaminated by Munn's input and Biotechnology Australia would not be entitled to exclusive patents. According to its agreement with CSIRO, it was well within its rights. As it demanded, the CSIRO's relationship with Munn was terminated.

Charles Morgan

UK computer research to switch to Europe, says policy review

London

THE gloomy predictions of Britain's information technology community were confirmed last week when the government finally rejected proposals for a £1,000 million, five-year research programme to succeed the Alvey project, which is nearing completion. The bulk of research in information technology will instead be done through the second phase of the European Community's £1,100 million ESPRIT programme, to which Britain has contributed £200 million. A modest national initiative is proposed to complement ESPRIT, for which the Department of Trade and Industry (DTI) will provide £29 million over the next three years, with the Science and Engineering Research Council (SERC) devoting £55 million over five years.

The Alvey programme was funded by £200 million of public money (through the Ministry of Defence, DTI and SERC), with industry providing £150 million. More than 200 industrial projects received support. In November 1986, a committee under Sir Austin Bide, former chairman of the Glaxo pharmaceutical group, recommended a continuation of the programme focusing largely on application of new technologies, with the government contribution increasing to £425 million.

The government's response to these proposals is contained in a white paper (policy document) published last week*, marking the completion of an extensive internal review of the DTI, instigated by the new secretary of state, Lord Young.

The white paper concedes that "the Alvey Programme has provided a good focus for the IT [information technology] research community". Researchers complain that not only is the proposed level of funding of the new programme inadequate for Britain to remain competitive, but the enforced switch from well-established domestic relationships between industrial and academic partners to a European programme, through the infamous Brussels bureaucracy, will result in damaging discontinuity.

The white paper signals a shift towards support for longer-term collaborative research and away from 'near-market' initiatives, which the government believes industry should itself finance. In line with stated government policy, the DTI will seek to encourage more collaboration between the industrial and academic sectors and between individual companies. Collaboration will be achieved, says the white paper, through European Community programmes and domestic initiatives such as the LINK programme (which was announced more than a year ago but which

has still not yet seen the light of day).

Also announced is the DTI's contribution to a national programme of research into high-temperature superconductivity: a total of £8 million to be made available over three years, but there is no news yet about how or to whom.

The review has little direct effect on the activities of the British National Space Centre, still part of the DTI despite its wish for autonomy. The white paper reiterates that no increase is proposed to the DTI's present contribution of £70 million to the space budget. But space minister Kenneth Clarke has been canvassing in-

dustry on its priorities for space research and the status of the space centre is being reappraised in order to allow closer links with the industrial community. An announcement on the future role of the space centre is expected before a replacement is found for the director-general, Jack Leeming, who retires at the end of next month.

To strengthen links between business and education, the government proposes that 10 per cent of teachers should each year "have the opportunity to gain some personal experience of the world of business". An extra £5 million will support advanced technology in schools and further education colleges, and The Teaching Company Scheme, which enables graduates to undertake projects in companies, will be expanded.

Simon Hadlington

*DTI — the department for Enterprise. HMSO, £5.00.

DNA fingerprinting database to finger criminals

San Francisco

THE world's first computerized data bank of 'DNA fingerprint' information on convicted criminals is now being planned. The California attorney general's office has begun studies to determine the best methods for collecting and storing data, and expects the database — which will be used to identify and prosecute repeat offenders — to be on line in 3–5 years. But questions over how the data will stand up in court may delay the database's applicability.

State forensic scientists are investigating several methods for analysing samples to be entered into the database, and they are unlikely to select the method developed by Alec Jeffreys of the University of Leicester (see *Nature* 317, 818; 1985), which has been used successfully in immigration and forensic cases in the United Kingdom. The Jeffreys method uses probes for hypervariable minisatellite DNA, and produces a complex pattern of bands that may be difficult to store in a database and compare reliably to newly run samples. Data storage and comparison will be simpler with the single-locus or dot-blot approaches, according to Steve Helsley, chief of forensic services in the California attorney general's office.

The dot-blot technique is considered less labour-intensive, works on the degraded DNA of forensic samples and requires only 10 nanograms of DNA, an amount obtainable from a single hair root. The 0.1–1 micrograms of intact DNA necessary for single-locus analysis is obtainable only in 60 per cent of the cases, according to John Winkler, president of Lifecodes, a New York company that specializes in DNA analysis.

Henry Erlich, director of human

genetics at Cetus Corporation, which developed the polymerase chain reaction and the dot-blot technique, says the company will soon have enough probes for informative polymorphic sites to offer identification of an individual with less than a one-in-a-million chance of a false positive result.

Ideally, the DNA samples for the database would be analysed in several different ways, says Helsley, because the technique used in a particular case may be dictated by the amount and condition of the DNA available for analysis.

Whatever method is chosen by California, defendants should have a source for an independent alternate test. Single-locus analysis is performed by both Lifecodes and Cellmark, a Maryland-based division of Imperial Chemical Industries (the British company that owns the Jeffreys probes), and Cetus plans to market a kit that should enable any laboratory to perform the simpler, dot-blot analysis.

Although DNA data have been used as evidence in criminal cases in at least five states, no case based on DNA evidence has resulted in a conviction, says Helsley. State Attorney General John Van de Kamp is not ready to have DNA go to court in California, because he doubts whether DNA forensic analysis has been sufficiently validated to pass the Kelly-Frye requirements, which state that there must be scientific consensus on the reliability of a forensic technique before the evidence is admissible in court.

The first entries into the database will come from the more than 5,000 blood and saliva samples collected from convicted sex offenders in California in the past five years.

Marcia Barinaga

Visas for scientific congresses

SIR—In the summer of 1986, the secretary general of the European Congress of Pathology, Dr J. Stejskal, invited me to take part in the congress, to be held in Prague in September 1987. He assured me that my being an Israeli would pose no problems in obtaining a Czechoslovak visa. I therefore registered and was told by the congress secretariat that I should obtain a visa in Vienna.

Having arrived in Vienna a week before the congress, I went several times to the Czechoslovak consulate and travel agency and telephoned Prague, but I was unable to obtain a visa and so could not participate in the congress.

This occurrence might be regarded as unimportant. After all, international congresses can be successful even if a number of scientists are excluded. No great harm was done, fruitful scientific exchange could still be accomplished and the progress of science was not appreciably disturbed.

In some respects the same reasoning could be applied to the burning of books and banning of unorthodox art and opinions. Books can be reprinted and banned art can find alternative outlets. Honest scientists and others who care for humanitarian ideals abhor such practices and resist becoming passive partners in discrimination of scientists because of nationality, race or creed.

I propose that all scientists who organize international congresses and meetings should make it a rule not to hold meetings in countries that practise discrimination. Definite promises from the governments concerned should be a condition for accepting invitations from such countries, and broken promises should be publicized in scientific journals in order to avoid further infringements of the rules.

MOSHE WOLMAN

Tel Aviv University,
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J. STEJSKAL REPLIES—I have read Professor Wolman's letter with regret and I understand his bitterness. We included him among the invited speakers for our congress because of his international reputation and his good relationship with Czechoslovak scientists and we did our best to enable him to come.

There are no diplomatic relations at present between Israel and Czechoslovakia, so that visas are dealt with by embassies in a third country, which complicates matters. For this reason, our secretariat added to the registration documents a note recommending that Israeli citizens should make the necessary application to the Czechoslovak Embassy

in Vienna as soon as possible. This recommendation was followed by other Israeli participants, who arrived in Prague without difficulty.

Unfortunately, however, Professor Wolman took his documents to the Czechoslovak consulate only a few days before the congress, which was deemed too late by the consular official concerned. Intervention by my secretariat was ineffectual, even though our invitation to Professor Wolman had been approved several months previously by the Czechoslovak Ministry of Foreign Affairs.

Although it is hard to understand why the simple act of stamping a passport should take longer than a few minutes, long intervals seem nowadays to be needed for international administration in spite of computers and other sophisticated devices. The British consulate in Prague takes two weeks to grant a visa to Czechoslovak scientists wishing to attend a congress in the United Kingdom, and the US and French consulates take three and four weeks respectively.

J. STEJSKAL

(Secretary General)

XIth European Congress of
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Touch of blackmail?

SIR—Serious discussion of the epistemological basis of scientific research is welcome. But to be useful it must be tolerably competent, which the recent article by Theocharis and Psimopoulos (*Nature* **329**, 595; 1987) is not.

The most serious flaw is the consistent perception of any sceptical or constructivist deviation from absolute inductivism as "epistemological anarchism". This is absolutism with a vengeance. One unfair remark in this vein is the assertion that "endorsement of the antitheses [to the orthodox realist thesis] saves one from the painstaking effort of discovering new truths", implying that epistemological sceptics do not or cannot undertake serious research leading to reliable knowledge. But what of John Ziman's (I should have thought respectable) view that the products of scientific enquiry, while as reliable (= true?) as we can possibly make them, must always remain open to revision?

The logical development of the whole article rests on the authors' assumption that whatever is not absolutely (timelessly, objectively) true must be untrue; that, however, is not supported by current philosophy, and is contrary to the assumptions underlying open scientific enquiry. This mistake invalidates the conclusions of the article, including the proposed

remedy for the under-funding of science, namely that scientists should "explain adequately how they propose to make new and fruitful discoveries".

Even the potentially interesting question of the psychological effect of the antithesis is treated shabbily: "If one believes that there exists no objective truth . . . it is highly unlikely that such a person would make any new discoveries (funding agencies take note)."

The touch of blackmail here sums it up: no treatment is too harsh for heretics.

NICHOLAS J. SELLEY

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Light ring grounded

SIR — John D.G. Rather's dismissal (*Nature* **329**, 480; 1987) of astronomers' protests (*Nature* **326**, 125; 1987) about the launching of the Eiffel Tower's light ring amply illustrates the cavalier barbarism of its proponents in relation to astronomy, in marked contrast to the sensitive expression of their idealism for their own science.

Unlike Venus and the Moon, the light ring would move rapidly relative to the stars. Thus astronomers could not point telescopes away from it without the risk that it would later interfere. It would have a low ratio of mass/cross-section and would be asymmetric, so, because of solar light and wind pressure and the variable effects of atmospheric drag, its orbit could not be reliably calculated much in advance. It would not be 'easy' to avoid. Because it would be visible as an extended object, the solid angle of space which it would sweep out makes the probability that it would have serious effects very significant during, say, a year's passes above the horizon, and these effects would not be 'improbable'.

A point which I have not seen addressed by its advocates is how the 'rubber' structure (or others like it) will look if, on launch, part of it fails to inflate, or if it deflates during orbit — perhaps it will look like a component in the widely broadcast advertising campaign which you advocate in your editorial (*Nature* **329**, 471; 1987).

Astronomers have demonstrated their commitment to the exploitation of space by participating in numerous space programmes. What celebrates the promise of space more — to launch a 'symbol of idealism and inspiration' which at the same time reduces our opportunity to understand our Universe or carefully and responsibly to use the near-space environment for the benefit of mankind?

PAUL MURDIN

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Keeping astronomers in the dark

Making sure the night sky stays dark near astronomical observatories makes good sense. For radioastronomers the problem is more difficult, but the solution is the same.

Tucson, Arizona

ANYONE who lives in a modern city or its suburbs will be quite mystified by the multitude of books on amateur astronomy meant to guide the novice around the spectacular sights of the night sky. The brightest stars in the best known constellations may be visible, but the Pleiades will be little more than a blur, while the Orion nebula and even the Milky Way might as well be fabrications. To such a city-dweller, the night sky seen from a dark and remote spot can be a breathtaking surprise, and may cause a sudden understanding of why some people devote their lives to observing the stars.

But there is more than social psychology to worry about. Astronomers at the big observatories are everywhere finding that city lights are creeping up on them, restricting the area of sky that can be usefully observed to the higher altitudes, and making it more difficult for instruments to pluck faint objects out of the background. The Mount Wilson Observatory, in the mountains near Los Angeles, has already been made useless; the night sky there is artificially five or ten times brighter than the natural sky, and this 'light pollution' is aggravated by real pollution, for dust and foreign gases also scatter and absorb light.

Lately, astronomers have been retreating to higher and more distant mountain tops, both to escape civilization and to get above as much of the atmosphere as possible. Three of the most important modern observatories are at Cerro Tololo in the Chilean Andes, on Mauna Kea in Hawaii and on La Palma in the Canary Islands. But even with continuing expansion, these sites cannot accommodate the world's demand for observing time, and efforts to forestall and reverse the deterioration of observing conditions at other observatories are essential.

At the Kitt Peak National Observatory, about 50 miles from Tucson, Arizona, there is some success to report. Since 1972, the city of Tucson has enacted a number of local ordinances governing the type of street lighting to be used, and the brightness and pattern of lights for store displays and advertising, which require some lights to be turned off at night. Astronomers have been astute in allying their good cause with others, such as energy conservation, and have found the electric utility companies co-operative in recommending higher-efficiency

shielded lighting to their customers.

Some of the changes seem absurdly simple. Installing lights at the top of a billboard pointing down rather than at the bottom pointing up makes a huge difference, as does a newer regulation requiring the background of advertising posters to be darker than the lettering. Local ordinances mean advertisers and shop-owners must restrain their natural extravagance, but if the rules are consistently applied nobody seems to object.

The result of this cooperation between the astronomers and the city officials is that artificial light at present makes up only about 8 per cent of the night-sky brightness at Kitt Peak. And some of that comes from Phoenix — 100 miles away, but a bigger city than Tucson — which has also recently enacted light-control measures. So while astronomers are pleased with their successes, they are fighting a growing trend, especially as Arizona is working to attract new industries and a younger population.

Big astronomy has come more recently to Mauna Kea, and scientists there have been able to work with the island government as the observatory has grown. In some ways their task has been easier than at Kitt Peak: energy conservation is already a public concern on Hawaii, and the observatory, having provided jobs and money to an economy which depends largely on tourism, is regarded as an important asset to the island.

That optical astronomy is a visible, benign science which generally enjoys good public relations has contributed to the success of light-control programmes. Radioastronomers are not so lucky. Their science is more mysterious to the layman, and radio interference can reach them from around the world. Any attempts by scientists in the United States to protect and regulate certain parts of the radio spectrum must work their way upwards through the National Science Foundation (NSF) to the Federal Communications Commission (FCC) and the National Telecommunications and Information Administration (NTIA), and thence into the international arena.

Radioastronomers cannot directly guard their own interests, and the FCC and NTIA are said to be less sympathetic to scientists than to the communications industry, which regards vacant parts of the electromagnetic spectrum as the mining industry regards the national parks.

Radioastronomers would like to maintain clear spots roughly in every frequency decade of the spectrum, along with a few particular reservations for important radio lines (mostly of hydrogen). But last autumn a World Administrative Radio Conference made new allocations, principally for users of mobile radio equipment, of frequencies near 1.6 GHz, and recommended a complete review of all allocations in the 1-12 GHz range.

NSF, which represents the astronomical community in international dealings, is gearing up to defend itself not only against the global communications industry but also against the suspicions of some other countries that the protection of clear bands is really being promoted on behalf of the owners of spy satellites.

Although global negotiations on the use of the radio spectrum are beyond the direct reach of astronomers, some local concerns have been dealt with more directly and with consequent success. The radio telescopes at Green Bank, West Virginia, have been protected by the establishment of a 13,000 square mile Radio Quiet Zone, within which radio operations are strictly controlled. At the Very Large Array, near Socorro, New Mexico, there is no comparable formal protection, but the site is remote and the few neighbours (the White Sands Missile Range and the US Forest Service) have been friendly.

Technical ingenuity can also overcome some conflicts. It is possible for radioastronomers to share a frequency with a local user who agrees to switch his equipment off for perhaps the first 200 milliseconds of every second. Interferometric observations using several telescopes in parallel are less vulnerable to local interference because the methods of data analysis tend to remove small-scale disturbances.

Terrestrial pollution, physical and electromagnetic, threatens astronomy of all kinds. An optimistic answer is to put astronomy in space, but even with the Hubble telescope soon to be in orbit and plans for space-based radiotelescopes widely discussed, no more than a tiny fraction of the observations that need to be done will be made from outside the atmosphere. But astronomers can achieve a great deal through their own efforts, and they are fortunate to be defending a science which has natural popular appeal and which can be explained in simple terms with relative ease. **David Lindley**

Supercooled water

Approaching the limits

C.A. Angell

WATER is famous for its many peculiar properties. Outstanding among these are the density maximum seen at 4°C at normal pressure, and the increase in fluidity and diffusivity which occur with increasing pressure up to 150 MPa before reverting to the normal behaviour at higher pressure. Prielmeier *et al.*¹ now add a new twist to the latter anomaly. They show that there is a striking difference in the magnitude of the anomaly when the property studied is rotational, as opposed to translational, diffusion. Their work, which gives new information on the provocative power-law temperature dependence of transport properties in water, raises a host of issues. In particular, their findings resonate with two recent developments concerning liquids and vitrification.

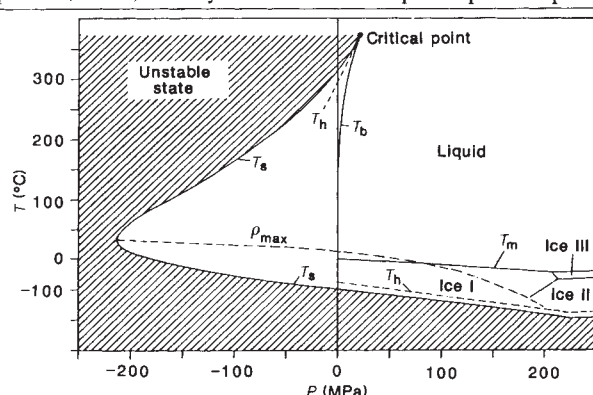
First, the transport properties, such as viscosity η and self diffusion D , of many simple molecular liquids and ionic solutions have power-law temperature dependences of the form $(T/T_0-1)^{\gamma}$ in the low-to-medium (0.001–10 Pa s) pressure regime. In particular, the exponents γ for viscosity are close² to the value 1.78 predicted by recent mode-coupling theories^{3,4}. (These theories predict vitrification — viscosity approaching infinity — at about 90 per cent of the melting point of known glass-forming liquids and about 1.3 T_g , where T_g is the measured glass transition temperature.)

Second, water itself, which had seemed for a long time to be unvitrifiable by liquid quenching methods, can indeed be vitrified using small sample techniques⁵. It has a very weak glass transition⁶ at 136 K. Before relating these findings to the work of Prielmeier *et al.*, note that for low-pressure water, the power-law description is clearly superior to the rival Vogel–Fulcher law, $\eta = A \exp B/(T-T_0)$, whereas the distinction is marginal for most liquids in the low-viscosity regime.

The power-law exponent for the low-pressure diffusivity in water is 1.8 according to the new data of Prielmeier *et al.*. The agreement with the mode-coupling predictions is a little unexpected as the detailed theory is derived for the relatively close-packing atomic liquids. (The first attempt⁷ to use mode coupling to describe diverging viscosities, however, was made for the case of water.) As expected from the relation between diffusivity and viscosity, $D \approx \eta/T$, the exponent for

diffusivity must be greater than that for viscosity, as is found; for the latter, the exponent is 1.64 (ref. 2) or 1.56 if data down to –35 °C are included⁸.

According to Prielmeier *et al.*, the exponent for water diffusivity increases with increasing pressure to values (2–2.5) characteristic of normal liquids¹. This is accompanied by a depression of the homogeneous nucleation temperature, at which pure water spontaneously crystallizes, and by an even more rapid depres-



Phase diagram for water. In the top right-hand corner water is stable as a liquid. To the left of T_b , the locus of boiling points, water can exist metastably as a superheated liquid in the absence of nucleation centres. To the left of T_h ordinary density fluctuations are sufficient to cause prompt spontaneous boiling because of 'homogeneous nucleation'. Similarly, below T_m , the locus of melting (freezing) points, water can exist as a supercooled liquid down to T_h , at which line homogeneous nucleation causes spontaneous crystallization. T_s is the locus (spinodal) of conjectured stability limits for water (the region is experimentally inaccessible because of T_h) beyond which there is no activation energy against nucleation. $\rho_{\max}$ is the locus of points (extrapolated) at which the density of water is maximum. (After ref. 1.)

sion of the temperature T_{MD} at which water exhibits its famous density maximum. Extrapolating to higher pressures, T_{MD} falls below the T_g s both of the power law and of the Vogel–Fulcher law reported by Prielmeier *et al.*

Prielmeier *et al.* interpret their data using the remarkable and, it seems, correct conjecture⁹ of Speedy (one of the co-authors) concerning the limits of mechanical stability of water. All equations of state predict for superheated liquids, both under pressure and under isotropic tension (negative pressure), a line of mechanical instability temperatures (or spinodal — T_s in the figure) beyond which there is no activation energy opposing the growth of fluctuations. Speedy deduced that for water this line of instabilities, rather than moving to ever more negative pressures with decreasing temperature (as expected for normal liquids from the van der Waals equation), must reverse direction at the point of

intersection with the (extrapolated) line of density maxima (see left hand end of figure). The reversed spinodal reemerges at a positive pressure some 46 °C below the freezing point of water and runs parallel to the observed homogeneous nucleation temperature up to about 200 MPa. In Speedy's picture, the origin of the diverging heat capacity and compressibility which make supercooled water so extraordinary, lies in the rampant density fluctuations that occur as one approaches the spinodal. It would be easy to disregard such a scheme as too imaginative were it not for the fact that its key prediction, that the tensile limit for isotropically stretched water at 35 °C should be –200 MPa has now been confirmed in detail by computer simulation studies (A. Geiger, personal communication). A second study at lower temperature to prove the reversal of the tensile limit has yet to be performed.

If water is indeed approaching a thermodynamic singularity on supercooling, then the same general mode-coupling arguments long used to link viscosity to thermodynamic divergences near the critical point (more appropriately, near a spinodal point) can surely be applied to supercooled water and will yield power-law divergences as observed. What, however, can be said about the high-pressure regime where the line of density maxima, by extrapolation, would again cross the spinodal? One possibility would be to argue as Speedy does⁹, at least implicitly, that the spinodal would change direction again and remain as a low-temperature limit on the homogeneous nucleation temperature of the supercooled liquid at higher pressures. But as

water behaves normally in this regime — no density maxima, thermodynamic or other anomalies remain — should not all strongly supercooled liquids have mechanical stability limits?

Another, to me more attractive, possibility is to argue that the spinodal, like the density maximum, is becoming less clearly indicated — more smeared out — with increasing pressure, and that in fact both vanish below the glass-transition temperature. In the same range, a Vogel–Fulcher law becomes as good a description of the data as the power law and gives a more rational value of T_0 . This happens because the geometrical *raison d'être* for the density maxima and the spinodal, the low-energy open-network structure, is removed by the increasing distortion of the bond angles. Then the Kauzmann (or ideal glass-transition) temperature (which is obtained either from thermodynamic data by finding where the liquid and crystal entropies are equal, or from the Vogel–

Fulcher equation for enthalpy relaxation¹⁰) replaces the spinodal as the conceptual limit for the behaviour of supercooled liquids.

In the normal world of positive pressures, the spinodal limit on supercooling would be correct for only a few liquids (water, SiO₂ and some aluminosilicates) that have either overt density maxima or at least the inverse of normal relations between energy and volume, and even then only at low pressures. But the article¹ by Prielmeier *et al.* requires us to think beyond the familiar and ask whether other liquids could become like water when isotropically stretched.

Sulphuric acid, H₂SO₄ — the world's second most abundant molecular liquid (the annual production is about 10⁶ tonnes) — for instance, has two protons tetrahedrally disposed with respect to two centres of negative charge, like water. Does H₂SO₄ become water-like upon stretching? Computer simulations should quickly tell. The question is whether water is unique (as is often thought) or is just the most prominent member of a class of liquids distinguished by their particle packing geometry and/or energy-volume relations.

Where does this leave the glassy state of water as formed by liquid quenching at normal pressures? It leaves it in a somewhat heroic position, because although a few freakish glasses, including certain aqueous solutions, are thermodynamically stable as they vitrify, most glasses are unstable with respect to the crystalline state as well as with respect to their initially equilibrated (though supercooled) liquid state. Vitreous water, however, is evidently the stolid survivor of yet a third sort of instability, that of the liquid state itself: it exists only because of the slowness of the fluctuations which, on cooling towards the spinodal, would carry the system right 'over the edge' of the liquid free-energy surface, $G(T, P)$, into a limbo from which it would emerge as a crystal for lack of other nearby free-energy surfaces.

The liquid-quenched glass appears to be remarkably similar in thermodynamic properties, structure, the kinetic stability against crystallization, to the substance formed by deposition from the vapour. This is consistent with the notion that, in disordered arrangements of water molecules which minimize the energy with

respect to bond bending, there are not many options. Likewise it is consistent with the finding, implied by the very weak glass transition, that when the supercooled liquid state is entered from the glass, there is very little increase in configurational degeneracy with increasing

temperature. In this respect, vitreous water and the related compound vitreous SiO₂ indeed have much in common. □

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Fungal genetics

Hazards in sexual transmission

John Fincham

FOR several years now, DNA-mediated transformation of filamentous fungi has been a routine procedure, particularly in the species *Neurospora crassa*^{1,2}. One of the questions arising from studies on such transformants in filamentous fungi is why so many of them are bad at transmitting their transformed character through sexual crosses. The special features of the *Neurospora* sexual process have enabled Eric Selker and his colleagues at the University of Oregon, who report their results in a recent issue of *Cell*³, to discover one genetic mechanism for this disruption in the transmission process.

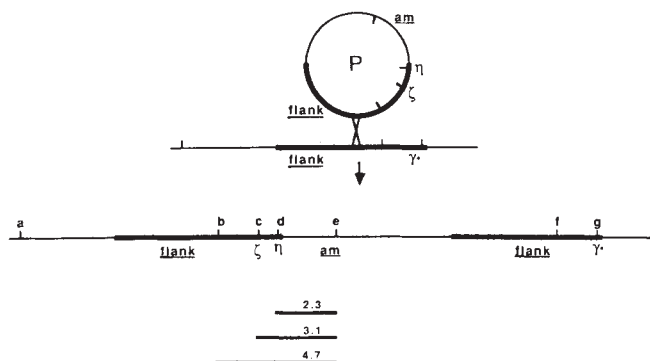
Transformation has generally been detected as restoration of a gene function to mutant strains initially lacking it by reason either of a crippling point mutation or a gene deletion. In *N. crassa*, the efficiency of transformation is not greatly dependent on the nature of the vector in which the transforming DNA is cloned. A stably self-replicating plasmid vector is not yet available in this species, and all stable transformants seem to be caused by integration of DNA into a recipient chromosome. Chromosomal integration frequently occurs at loci without obvious homology to the transforming sequence — in striking contrast to the situation in yeast (*Saccharomyces*), where integration almost always depends on homology. Thus, in cases where a point mutant is used as recipient strain, many or most *Neurospora* transformants will have at least two copies of the gene in question — a mutant allele at the normal locus and

one or more ectopic wild-type gene copies at non-homologous loci.

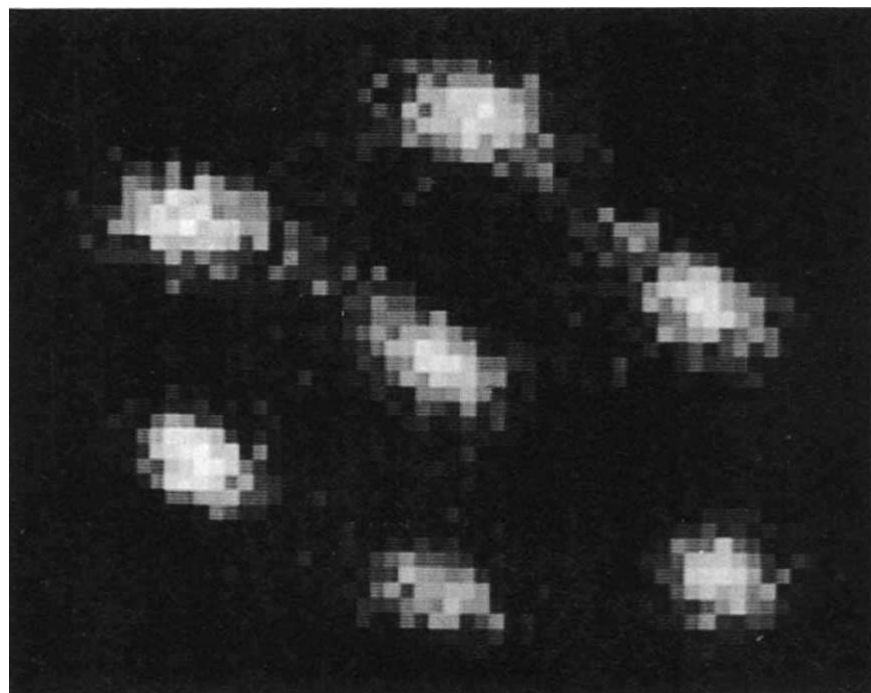
When vegetatively stable transformants are crossed back to the untransformed mutant, one often finds far fewer than the expected 50 per cent frequency of phenotypically wild-type ascospores and sometimes none at all. Mary Case of the University of Georgia, one of the pioneers in developing the *Neurospora* transformation system, recently reported⁴ that ectopic copies of the *qa-2*⁺ (catabolic dehydroquinase) gene were transmitted through meiosis but sometimes with changes in the sizes of the restriction fragments within which they are located. Even when (as judged by Southern blot analysis) the sequences came through unchanged, their phenotypic effect was often lost. No explanation could be offered for this result other than the remote possibility of high-efficiency meiotic conversion of the ectopic wild-type alleles to the sequence of the mutant allele still resident at the normal *qa-2* locus. Because even homologous meiotic gene conversion is rare in *Neurospora*, frequent conversion operating between non-homologously located alleles would be surprising.

In their new work³, Selker and colleagues present compelling evidence, again from *Neurospora*, that disruption, manifesting itself in various ways, can result from the presence of duplicated sequences in premeiotic haploid nuclei. Selker set up his duplication by transforming an *am*⁻ (glutamate dehydrogenase-less) deletion mutant with a plasmid carrying

Fate of transforming DNA integrated into *N. crassa*. Heavy lines, homologous sequences between plasmid (P) and transformant. The chromosomal region γ^* is roughly 80% homologous to the ζ - η region. a-g, *Bam*HI restriction sites; below, *Bam*HI-generated fragments in genomic DNA of the transformant, kb. (From ref. 3.)



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Each 'blob' in the figure is a single magnesium ion caught in an ion trap by H. Walther and colleagues at the Max Planck Institute for Quantum Optics. They and a group at the National Bureau of Standards at Boulder, Colorado, separately report in a recent issue of *Physical Review Letters* (59, 2931–2934 and 2935–2938; 1987) the unexpected observation of a phase transition in a cloud of 2–50 ions cooled by a laser in a dynamic 'Paul' trap. The seven ions in the figure, for example, make a planar crystal, ordered by their mutual electrostatic repulsion, with a separation of about 23 μm . Their large apparent size arises from their micromotion, caused by the radiofrequency field of the Paul trap, about their equilibrium position during the 40-s 'exposure'. The Boulder group has performed conventional spectroscopy on 'pseudomolecules' of one or two trapped mercury ions, to find their molecular 'constants' such as vibrational frequency. $\square$

both *am*⁺ (as a selectable marker) and an eight-kilobase *Neurospora* sequence from a 5S-RNA gene flanking region (see figure). This *flank* region was the only sequence shared by the plasmid and the recipient genome. The authors found one transformant in which the plasmid had been integrated by homologous recombination between the chromosomal and plasmid-borne *flank* sequences, so that these sequences were present in the transformant as a tandem duplication with *am*⁺ and the rest of the plasmid sequence between them in single copy. When they crossed this transformant back to *am*[−], only a few progeny retained the *am*⁺ marker — but these included all those that seemed to have undergone structural alterations in one or (usually) both of the duplicated sequences. These alterations included extensive cytosine-methylation, even though the *flank* sequence is not normally methylated.

Some progeny also show new *flank* restriction fragments that could not be explained by methylation and must have been caused by deletions or other rearrangements within the *flank* sequences. Significantly, the nearby non-duplicated *am* and plasmid sequences generally have no such alterations. The

more numerous *am*[−] progeny do not show any *am* or plasmid sequence at all; many of them must have excised all their newly integrated DNA, probably by meiotic recombination between the tandemly duplicated *flank* sequences. However, the remaining single copy of the *flank* sequence is sometimes modified.

The special features of the *Neurospora* sexual process allowed Selker to deduce that these disruptive events occur in haploid nuclei shortly before nuclear fusion and meiosis, thus allowing him to coin the arresting acronym RIP (for rearrangements induced premeiotically). Each fruit body (perithegium) results from the pairing of two haploid nuclei, one from each parent, but before nuclear fusion there is a phase in which pairs of nuclei divide in synchrony within dikaryotic ascogenous hyphae, which fill the developing perithegium. Premeiotic DNA synthesis takes place in the tips of the ascogenous hyphae just before nuclear fusion, followed immediately by meiosis and ascospore formation.

Selker finds that the same distinctive pattern of methylation and other disruption is generally shared by two of the four members of a meiotic tetrad, showing that RIP must occur before premeiotic DNA

replication. Tetrads from the same perithegium mostly show different (though possibly related) patterns, but in a few cases the same pattern is shared by two tetrads. From this Selker and colleagues conclude that 'RIPing' is not confined to nuclei immediately before fusion but could occur during proliferation of the dikaryon.

The discovery of the RIP effect may explain the disappointing performance of transforming DNA in sexual crosses. One may predict that, where transformation results from ectopic integration, the transforming sequences will escape RIPing only when the corresponding sequence in the recipient genome is deleted, and even then only when the transforming sequence is integrated in only single copy. Among transformants caused by homologous integration, only those resulting from sequence replacement (conversion) should be stable through crosses; those from crossover integration, with the recipient mutant allele retained in tandem with the transforming allele, will undergo RIP, as in Selker's experiment. But what of the silencing of Case's ectopic *qa-2*⁺ genes without evident sequence rearrangement? Maybe they suffered internal deletions too small to show as changes in fragment size, or maybe they were silenced by extensive methylation.

As to the mechanism of the RIP phenomenon and why it is confined to the ascogenous hyphae, nothing can yet be said with confidence. One could speculate that the dikaryotic cells of the ascogenous hyphae already have some of the components of the synaptic and recombinational apparatus of meiosis, waiting for homologous pairs of chromosomes to operate on. Normally these will be present only after karyogamy, but duplicated sequences already present in the prefusion nuclei might be made to synapse and be subjected to premature recombinational endonuclease activity. These events could trigger a sequence of fairly chaotic repair processes.

The relevance of methylation to such a scheme is still unclear — could it have some role in meiotic recombination or DNA repair? Another question concerns the apparent immunity to the RIP effect enjoyed by such normally repetitive sequences as ribosomal DNA or 5S-RNA genes. The RIP phenomenon may be relevant to the understanding of meiosis as well as to the properties of fungal transformants. $\square$

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Molecular immunology

MHC gene in cytomegalovirus

Don Wiley

ON page 269 of this issue¹, Stephan Beck and Barclay Barrell report the discovery in human cytomegalovirus (HCMV) of a gene similar to those encoding class I major histocompatibility (MHC) molecules in vertebrates. HCMV, a herpesvirus, causes widespread, subclinical infections in humans and significant illness and death among immunosuppressed patients such as those receiving organ transplant² or suffering from AIDS³. Because the class I histocompatibility molecules of an organism are essential in the presentation of viral antigens and in the recognition of infected cells by cytotoxic T-cells⁴, the presence of a class I gene in a virus raises interesting questions about its activity.

In higher eukaryotes, class I genes encode two types of cell surface glycoproteins: polymorphic cell surface glycoproteins, which are markers of immunological 'self' and are expressed on nearly all cells (HLA-A, B, C products in humans, H-2 K, D, L products in mice); and non-polymorphic glycoproteins, sometimes called differentiation antigens (such as Qa and Tla products in mouse) which have limited temporal expression. All cellular class I genes encode a glycoprotein of relative molecular mass (M_r) about 45,000 (45K), which has a carboxy-terminal membrane anchor and non-covalently associated with the 11K protein β_2 microglobulin (β_2m).

The new data of Beck and Barrell in this issue show that the sequence of the HCMV gene HCMV-H301 has many of the features of a class I gene. It encodes a hydrophobic leader sequence, followed

by a 350-amino-acid protein that contains a carboxy-terminal hydrophobic membrane anchor and a 25-amino-acid cytoplasmic domain. The three class I extracellular domains, α_1 , α_2 and α_3 , can be recognized in the sequence by their significant similarity to published class I sequences, including, for example, the conservation of disulphide-linked cysteines.

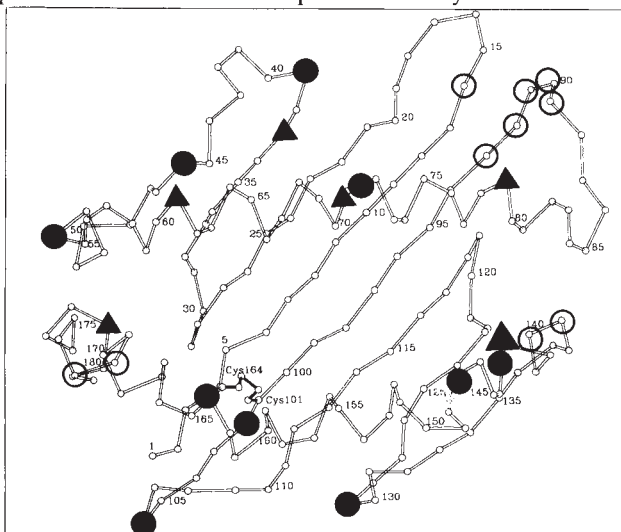


Fig. 2. Proposed antigen-binding site of HLA-A2. Changes as for Fig. 1. Large triangle, insertion of 11 amino acids.

No introns, which separate domains in cellular class I genes, are found in the HCMV gene, suggesting (among other possibilities) that the viral gene may have originated from a cellular messenger RNA.

Grundy and co-workers have shown previously⁵⁻⁷ that HCMV binds β_2m , the protein that is normally found associated with the class I antigens. It is tempting to suggest, as Beck and Barrell do¹, that the HCMV class I-like gene may be responsible for this binding. Two viral envelope proteins, of M_r 36K and 65K, can be

immunoprecipitated with an antibody against β_2m ⁵⁻⁷. If seven or eight of the thirteen potential glycosylation sites of the HCMV protein, which has a predicted unglycosylated size of 45K, are used, the resulting glycoprotein might have an apparent M_r of 65K. There is no direct evidence, however, that the HCMV gene encodes the β_2m -binding protein observed by Grundy and co-workers⁵⁻⁷.

Comparison of the sequence from the HCMV gene with the three-dimensional structure of human HLA-A2 (ref. 8) does not make matters much clearer. Of the residues implicated in binding to β_2m (see Table 2 of ref. 8 and Fig. 1), those in the α_1 and α_2 domains are only about 50 per cent conserved, and those in α_3 even less (although the only salt bridge is conserved). So, although the HCMV protein can bind β_2m , the strength and geometry of the interaction may not be identical to the binding on normal (cellular) class I molecules. One murine class I molecule is known (D^b) that can be expressed at the cell surface in the absence of β_2m ¹⁰. Thus, relatedness to a class I molecule does not mean that binding to β_2m is a foregone conclusion.

What the comparison with the known structure of HLA-A2 does show is that, although many of the novel potential carbohydrate attachment sites, amino-acid insertions and deletions are found on loops where they could be accommodated without altering the structure (see Fig. 2), five new potential glycosylation sites and four insertions, one 11 amino-acids long, are found on the α -helices flanking the proposed foreign antigen-binding site⁹ of class I molecules. If these sites are glycosylated (and for this molecule to match the β_2m -binding protein in size it must be heavily glycosylated), the surface formed by the α -helices, which is the proposed site of recognition by T cells¹, would probably be covered with oligosaccharides, and would in any case be structurally altered by the insertions. Such gross structural alterations would probably destroy its capacity to bind antigenic peptides, and might prevent the molecule from being recognized as a class I molecule at its T-cell receptor recognition site.

Why does HCMV have a class I gene? The gene could of course be a functionless retroposon, and its presence in the genome of a virus that binds β_2m a coincidence. Assuming that that is not the case and that the gene is expressed and has a function, is it the β_2m -binding protein? Analysis of the sequence suggests that this is possible, but not certain. If it does bind β_2m , what could its function be? Grundy *et al.*⁵⁻⁷ and Beck and Barrell¹ suggest it is

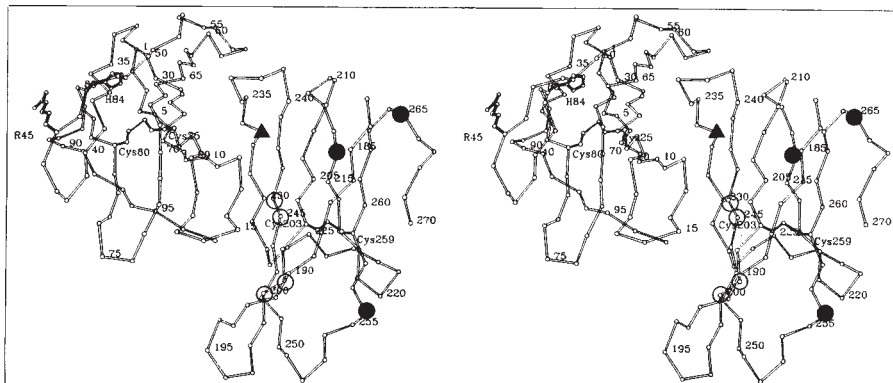
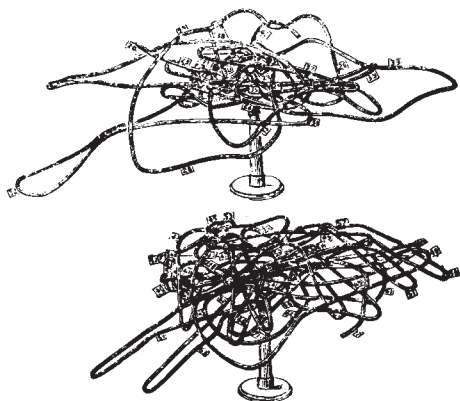


Fig. 1. Stereo pair of the α_3 region of HLA-A2 and β_2 microglobulin. Changes in the sequence of the class I-like HCMV gene are marked. Closed circles, potential glycosylation sites; triangles, amino-acid insertions of 1-3 amino acids; open circles, deletions.

100 years ago

A MODEL OF AN EARTHQUAKE



Prof. Sekiya describes a very curious and remarkable model he has made to exhibit the manner in which a point on the earth's surface moves during an earthquake. The motion which is recorded at an earthquake observatory is a prolonged series of twists and wriggles of the most complicated kind, so that the path pursued by a point on the surface of the soil has been aptly compared to the form taken by a long hank of string when loosely unravelled together and thrown down in a confused heap. From *Nature* 37, 297; 26 January 1888.

involved in virus-cell attachment.

Equally fascinating would be some involvement in the cell-mediated immune response of the host. One possibility would be that it might interfere with the induction of cell-mediated immunity, another (less advantageous to the virus) that it induces a cytolytic T-cell response that does not require the involvement of host class I molecules. Both these possibilities seem unlikely, because healthy humans respond to HCMV infection with a typical (host) class I MHC-restricted virus-specific cytolytic T-cell response.

Unfortunately, not enough of the basic questions have been answered yet for us to make a sufficiently informed guess about the meaning of the intriguing result of Beck and Barrell. But at the very least, if the gene encodes a protein with a useful viral function, we have an opportunity to study an example of a viral glycoprotein hijacked from host cells and converted through evolution to its own purposes. □

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Geochemistry

Archaean carbon and gold

E.G. Nisbet and T.K. Kyser

THE origin and distribution of CO₂-rich fluids deep in the Earth's continental crust is a taxing problem not only for metamorphic petrologists, but also for seismologists, igneous petrologists, those modelling planetary degassing and the history of the atmosphere and biosphere, those exploring for gold, and even the occasional astrophysicist. The debate centres on the question of whether these fluids are ultimately derived from reservoirs fixed near the Earth's surface (carbonate sediments, organic carbon and seawater-derived hydrothermal carbonates), originate from the mantle, or represent recycled mixtures of both. Groves *et al.*, on page 254 of this issue¹, conclude from the carbon-isotope composition of Archaean (older than 2.5×10⁹ yr) gold deposits that sea water alone is not the source.

In Archaean gold lode deposits, such as those in Canada and Australia, gold mineralization occurs with sulphides, often in quartz veins, which are enveloped by or associated with rocks rich in carbonate. The gold may have been carried into place by reduced sulphur complexes in low-salinity H₂O–CO₂ fluids. Where did these fluids come from? One hypothesis is that the fluids were derived by metamorphic degassing of crustal rocks²; alternatively, they may have magmatic sources³.

One facet of the debate about the origin of these fluids hinges on the interpretation of the carbon-isotope composition of the fluids. This is measured by the δ¹³C parameter, which gives the difference in the measured ratio ¹³C/¹²C from that in a seawater-derived standard, normalized to that standard. Variations occur because of fractionation, and negative values show that the sample is slightly depleted in ¹³C. For carbonates derived from sea water, δ¹³C ≈ 0‰. For reduced carbon δ¹³C ranges from –10‰ to –45‰, and the mean crustal value for oxidized and reduced carbon is –5‰ to –7‰.

Sea water

Most carbonates from gold mines appear to have δ¹³C values between –2‰ and –4‰. If the CO₂ that is now present in gold mines came from metamorphic decarbonation of seawater-derived carbonate, the δ¹³C value of the carbonate associated with the gold would have a small positive value. It is unlikely, therefore, that sea water alone is the source. On the other hand, the δ¹³C values of average crust (–6 ± 1‰), magmatic (about –5‰) and mantle (about –2‰ to –5‰) rocks

are generally comparable⁴, and none of these can be considered a unique source for the carbonate associated with the lode gold.

Burrows *et al.* suggest³ that the source for CO₂ in many carbonates associated with gold lode deposits was originally magmatic, originating from outgassing of melts intruded into the crust, because of the similarity in the range of δ¹³C values of gold-related carbonate to typical magmatic values. Groves *et al.* summarize¹ much of the available δ¹³C information — the scatter is large, ranging from 0‰ to –10‰ for gold deposits, considering the subtlety of the arguments based on the data — and conclude that sea water alone could not have been the sole source of carbon for the CO₂ in the metamorphic fluids. Some carbon, at least, must have been derived from a source with a negative δ¹³C value such as the mantle.

Degassing

In contrast to those who have proposed a direct magmatic source³, however, Groves *et al.* argue¹ that some CO₂ was introduced into the rock via major fault zones which were fed with CO₂ by metamorphic degassing. But in the spirit of compromise, they suggest that the original source of this CO₂ was in the mantle. This is interesting in the light of the arguments of those who have suggested that massive degassing of carbon from the mantle is taking place.

More generally, the debate illustrates our lack of knowledge of the Earth's carbon cycle. Carbon is degassed at within-plate volcanoes and at mid-ocean ridges, but most carbonate is added to new ocean floor by hydrothermal alteration. This CO₂, together with any organic matter added to the ocean floor, is carried down the subduction zones and acts as a source for the CO₂ degassed by volcanoes and perhaps even for diamonds. Javoy *et al.* argue⁴ that oceanic basalts also contain carbon with very low δ¹³C values (down to –25‰) and further that the δ¹³C value of CO₂ outgassed in magma erupted above subduction zones varies substantially with depth of origin and distance from the trench. The 'mantle' source of the subcontinental carbon could be variable and originate from subduction zones.

It is also possible that biological effects alter the δ¹³C value of 'mantle' carbon. Biological carbon is very light (δ¹³C ~ –30‰) and work in Archaean high-grade terrains has shown that rocks originally formed on the surface within the biosphere often constitute part of the deep continental crust. Perhaps this may be a

source of carbon with low $\delta^{13}\text{C}$ values in the gold mines, especially as bacteria may have lived and died in the hydrothermal systems that eventually degassed during metamorphism to give rise to auriferous fluids. Yet the discussions of carbon in carbonate from Archaean gold lode deposits usually ignore any biological effects.

The debate will continue: perhaps soon the paths of CO_2 movement will be mapped better and we will understand much more about the history of CO_2 degassing. The new work will be useful for

finding gold, which should be profitable. More than that, in revealing the way in which the Earth manages its carbon budget, the work will aid our understanding of the history of the biosphere and the continents. □

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Evolutionary biology

Learning in parasitic wasps

H.C.J. Godfray and J.K. Waage

ABOUT one out of every ten forms of metazoan life is an insect parasitoid, most of these being parasitic wasps. Insect parasitoids lay their eggs in, or on, the bodies of other insects which act as food for their developing offspring. Parasitoids and their hosts are small animals living in a very complex vegetational environment: the chances of finding hosts by random searching are remote. As a consequence, parasitoids have evolved a wide array of searching strategies, often making use of cues that indicate the presence of a host or a suitable environment for a host. Although it was once thought that these strategies involved innate responses of great specificity, evidence is now accumulating that parasitic wasps frequently learn from their experiences. On page 257 of this issue¹, Lewis and Tumlinson provide an excellent example of how wasps use their experience to improve searching strategies.

The commonest type of cues used by

parasitic wasps are chemical, although examples are known of the use of visual, vibrational and auditory cues (see refs 2,3 for reviews). Chemicals used by searching, parasitic wasps can be volatile attractants which lead them to areas containing hosts, or non-volatile arrestants, usually called 'contact' chemicals, which cause them to concentrate movement in these areas. Attractant chemicals are often produced by the host themselves, usually incidentally in their frass (excrement) or salivary secretions, but sometimes the wasps subvert the host's own chemical communication system by homing in on sexual or aggregation pheromones. Volatiles produced by sources associated with the host, such as its food plant, can also provide attractant stimuli, and some parasitoids are capable of sniffing out the infested plants in a crop, because of chemical changes in the plant caused by the host.

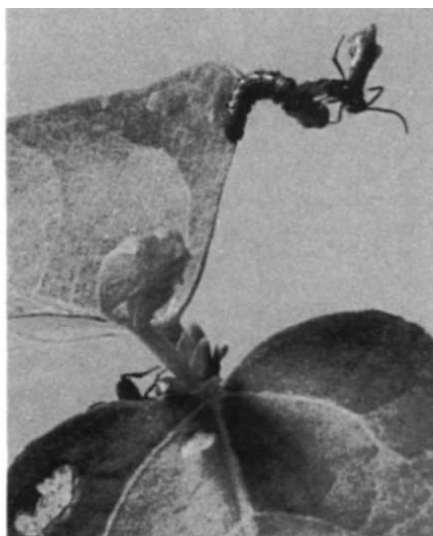
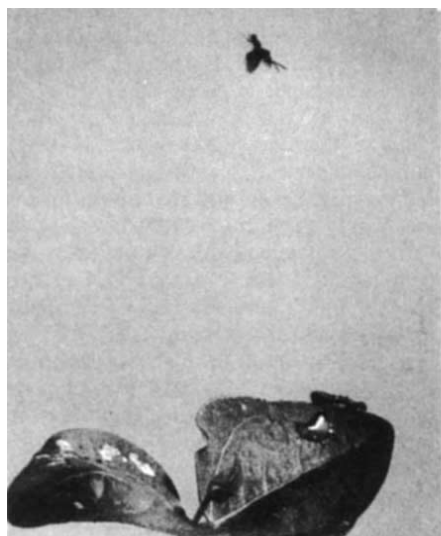
The value of certain stimuli to parasitic

wasps varies depending on local conditions. Imagine a wasp that parasitizes several different host species. The abundances of the host species will change both spatially and over time, so that an associational cue, such as the host's food plant, will vary in its information content. The wasp can obtain some information about the local distribution of hosts by noting the volatile stimuli in the environment where she developed. Perhaps this learning takes place in the larval stage, but it is more likely to occur immediately after the adult wasp emerges from the pupa. The wasp may also note which volatile chemicals are present in an area where it has found hosts, and subsequently follow these attractants in the hope that they will lead to further hosts.

There have been several studies on learning in parasitic wasps (see ref. 4 for review), one of the best being the work of Vet and her collaborators^{5–7} on parasitic wasps attacking *Drosophila* larvae, which develop in fungi and fruit. One species of wasp is normally attracted to the odour of decaying fungi, but if cultured on a yeast medium, the adult wasps show an increased attraction to the yeast odour characteristic of rotting fruit. The attraction is substantially increased if the wasps encounter and parasitize hosts in a yeast medium. This learning suggests that parasitoids can shift over several generations between the *Drosophila* faunas of fungi and fruits: the searching strategy is an example of a culturally transmitted trait.

In their paper in this issue, Lewis and Tumlinson describe for the first time an example of a wasp learning to associate volatile stimuli, not with its place of development or the host itself, but with the host's frass, a very strong indication of the presence of the host (see figures). The wasp innately recognizes a non-volatile compound in the frass and then associates it with any volatile present, which it uses as attractants in the future. Lewis and Tumlinson show that even completely alien substances such as vanilla can be used by the wasp. The wasp used in their study attacks a host with a wide variety of food plants. The most likely advantage of this behaviour is that it enables the wasp to learn the food plants that, at that time and place, are being most used by the host. □

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Left, female *Microplitis croceipes* wasp following an odour plume to locate the host caterpillar feeding on a bean plant leaf. Right, chemicals (first detected as odours in the air, later on plant surfaces) lead the wasp to sting its caterpillar host. As the wasp stings, an egg is oviposited into the caterpillar which will ultimately kill it. (Courtesy of Lewis and Tumlinson/Tim McCabe.)

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Cell biology

Muscle-bound bacteria and weak worms

Michael P. Sheetz

FOR myosin to function properly in muscle, its bipolar filaments must be assembled in ordered arrays. A major problem facing cell biologists is the control of local assembly of the filaments. Within non-muscle cells, the filaments are continually assembling and disassembling according to local signals. In muscle cells, however, myosin must be turning over continuously within a permanent array and, therefore, the cells must be replacing myosin subunits or whole filaments in an organized fashion. Two different approaches have now been applied to find out how this is achieved. The first is a classical genetic approach in which mutants of the nematode worm *Caenorhabditis elegans* with incorrect myosin assembly are isolated¹; and the second is by inducing expression of portions of the myosin molecule in bacteria². The differences in the information about myosin filament assembly provided by these two methods offer a nice contrast between genetic and molecular-biology approaches to a cell biology problem. Analysis of mutants reveals behaviours requiring molecular explanations, whereas the expression of pieces of the molecule gives information about the behaviour of specific domains of the molecule.

On the basis of the amino-acid sequences of the myosin molecules, it is believed that repeating stretches of residues in the tail of the monomers produce the tertiary structure responsible for their assembly into filaments^{3,4}. The conserved nature of these domains, and the fact that the assembly behaviour of myosin can be reproduced with proteolytically derived tail fragments, suggests that assembly is a function of the tail alone. There must be regulatory sites which when given the proper signal will modulate the assembly of bipolar filaments.

It is remarkable that Jim Spudich and collaborators now find² that expression of a fragment of relative molecular mass 56,000 (56K) of myosin tail from the amoeba *Dicystostelium* in bacteria forms filaments with a 14-nm repeat like native myosin from muscle. This result means it is now possible to test rapidly for the effects of various amino-acid substitutions and deletions on assembly of the filaments. As an indication of the speed of progress, smaller fragments of the tail have already been expressed which demonstrate that filaments can form from as little as 34K of the carboxy terminus of the myosin molecule⁵ (see figure).

As might be expected for non-muscle myosins, there is a site(s) that can regulate filament assembly and disassembly. In some non-muscle myosins, the phosphorylation of the regulatory light chain seems to be responsible for promoting assembly, whereas in *Dicystostelium* myosin it is dephosphorylation of the tail which promotes assembly. The phosphorylation

of this site occurs normally in the 56K and 68K fragments from bacteria but will not occur in the 34K fragment. This opens up the possibility of determining *in vitro* the portions of the myosin sequence involved in filament formation.

Is all the information relevant to the assembly of myosin filaments contained within the tail sequences? It is possible that physiological ligands could act through sites outside the tail to regulate filament assembly. It is difficult, if not impossible, to predict which sites on the molecule would affect filament assembly; therefore, the screening of mutants could uncover other physiologically important sites. *C. elegans* worms with weak muscles often do not form normal bipolar skeletal

Luis F. Leloir (1906–1987)

LUIS LELOIR, the outstanding biochemist, died on 4 December. One of his earliest contributions, which attracted immediate attention, was the discovery of glucose-1,6-diphosphate and its role as the cofactor for phosphoglucomutase. This served as a model for the proposal of a similar role for 2,3 bis-diphosphoglycerate for phosphoglycerate mutase by Cori, Sutherland and Pasternak. Then followed his main discovery, that of uridinediphosphate glucose (UDPG) and its role in galactose metabolism. Soon thereafter, Leloir and others found new reactions involving sugars, for example, in sucrose and lactose biosynthesis and in plant glycosides. In 1957 Leloir, with Cardini, discovered glycogen synthetase and that the enzyme used UDPG. He received the Nobel prize in chemistry in 1970, becoming, after Houssay, the second Nobel laureate in science from Argentina.

The discovery of the uridine nucleotide-sugar compounds by Leloir and others led to the isolation of other substances having either a different nucleoside moiety, such as adenosine, guanosine, cytidine and thymidine, or of derivatives in which the nucleoside diphosphate moiety is linked to compounds other than sugars.

Later, Leloir became interested in the role played by polyprenols in the synthesis of complex polysaccharides. Starting in 1970, he and his colleagues showed the presence in animal tissues of a microsomal acceptor lipid which accepts glucose residues from UDPG. He also showed that dolichol, a polyprenol from liver, accepts glucosyl residues from UDPG to form UDP and dolichol-phosphoglucose, and that this compound can transfer its glucosyl residues to proteins. These processes are operative in the synthesis of glucoproteins.

Although Leloir was born in Paris, his parents were Argentinians and he lived there all his life except for periods of study abroad. He studied medicine at the University of Buenos Aires. On graduating in 1932, he worked with Houssay at the Institute of Physiology, where his interest

in carbohydrate metabolism began. In 1936 he went to Cambridge, UK, to the laboratory of Sir Frederick Gowland Hopkins. On his return to Buenos Aires he worked on fatty acid oxidation with J.M. Muñoz.

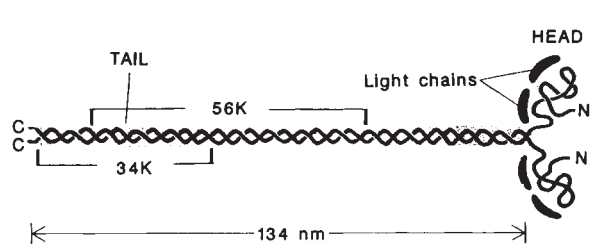
Houssay was dismissed by Peron in 1943; many of his students and colleagues, including Leloir, resigned in protest. Leloir went to the United States, where he spent some months with Cori in St Louis and then with Green at Columbia University in New York. Meanwhile, Houssay had obtained private support for a research institute for Leloir, so he returned to Argentina in 1945 to head the Instituto de Investigaciones Bioquímicas (Fundación Campomar).

A key point in Leloir's career and in his recognition at home, as Deulofeu pointed out in a publication in his honour in 1973, was his invitation to the now classical symposia on metabolism initiated by McElroy, in 1951, in Baltimore (many participants became Nobel prize winners), where he presented his work on UDPG.

The institute where Leloir's studies began was both modest and limited in resources, but was not limited in ingenuity, shown, for example, by the construction of a fraction collector using a toy train. As more students were attracted there, more suitable facilities were obtained. Leloir pursued his research with determination in the face of the most adverse circumstances, and he had an enormous influence on scientific development not only in Argentina but throughout Latin America. He was a modest man with a dry sense of humour, graced by charm and simplicity. I remember being with him in Buenos Aires in the late 1950s when he confided how at one point it had almost been necessary for him to choose between polo and science. Although his health had not been robust for several years, he attended the Institute daily until his death. Santiago Grisolia

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The myosin molecule, showing the tail, comprising two coiled α -helices, and the head (S1), comprising two globular portions each with two light chains. C, carboxy termini; N, amino termini. The S2 region is the part of the tail immediately behind the heads and extends into the heads.



muscle filaments¹. When some of these mutants were mapped to the *unc-54* locus, which is one of the four skeletal myosin genes in the worm, one mutant, e1152, is found to have only two adjacent amino-acid substitutions in the S2, or 'hinge', region of the myosin molecule (see figure). It is surprising that an alteration in the hinge would alter assembly. Such observations remind us that there are evolutionary complexities built into this motor which may yet take years to elucidate.

This latter thought raises a problem of excess which many cell biologists and biochemists are currently facing. Given a protein with 2,000 amino acids, all of which could be permuted 19 additional ways, there are 38,000 single-site mutations that could be formed, 1.5 thousand million two-site mutations, and so on. In other words, we cannot hope to study all the possible mutations of myosin.

Is there a way out of this dilemma? In the face of all the genetic information about the relevant amino acids in the myosin tail, it is easy to lose sight of what really does control bipolar filament assembly. In physical-chemical terms, the association constants of the subunits determine whether or not filaments will assemble. Because these association constants are

a function of ionic strength, pH and concentrations of other ionic ligands, it is important to relate findings to physiologically relevant conditions. Otherwise there is a risk of developing a complete subdiscipline of biochemistry on the comparative behaviour of altered myosins over a wide range of bizarre conditions. Ultimately, an understanding of the relative contributions of different amino-acid side chains to the energetics of filament assembly will be most useful in the educated design of new myosins and can come from *in vitro* expression studies. On the other hand, the analysis of mutants provides important clues about other aspects of the bipolar filament assembly process which could not have been predicted. The muscle-bound bacteria and weak worms provide complementary information which should reveal the molecular details of myosin filament assembly. □

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Astrophysics

A relaxed approach to galaxies

M. G. Edmunds

VIOLENT relaxation was first identified 20 years ago¹ as an important mechanism in the formation of galaxies, but since then surprisingly little progress has been made in elucidating the process. It is used to explain how galaxies formed far from equilibrium can rapidly redistribute their internal energy to become the persistent structures now known. Recent work² by S.D.M. White and R. Narayan shows that the known forms of galaxies are close to a constrained maximum-entropy state, something which had not been shown before. How galaxies approach this state is still an important issue to resolve.

Elliptical galaxies, and the old spheroidal bulges of spiral galaxies, show remarkably regular structures. The distribution of light is in general a very smooth function of radius from the

nucleus, and can be well represented by a simple functional form, with just a scaling parameter varying between galaxies. Such regularity of structure, maintained over long times, implies that the galaxies are at dynamical equilibrium with a well-defined distribution of energy among the stars. The problem is in finding out what the distribution is, and how it came about.

To reach an equilibrium, stars must interact to share out their energy. The simplest mechanism of 'relaxation', involving close encounters between pairs of stars, is far too slow at the typical densities of stars found in galaxies. The stars are too far apart, and form an essentially collisionless system, with two-star interactions having negligible effect except on timescales much greater than the age of the Universe.

But if a large system of stars starts from a state that is far from equilibrium and is allowed to collapse under self-gravity, the changing gravitational field efficiently redistributes the energy between the stars, while conserving the overall energy of the system. The effect is a collective one, and an individual star is affected not only by the overall smoothly varying field of the whole galaxy, but also by strong perturbations in this smooth gravitational field caused by transient clustering of groups of stars within the collapsing galaxy³. The collapse slows down as the stars gain enough kinetic energy to establish hydrostatic equilibrium, and the system settles into a long-lived state as the collective interactions die out. The stars experience chaotic gravitational accelerations for a short time, allowing redistribution on a timescale much shorter than the age of the Universe — violent relaxation.

Because the galaxy has reached a state of equilibrium with its energy shared out it might be thought that statistical mechanical arguments could predict what that state should be. If energy sharing implies a single temperature for the stellar motions, with a Maxwell-Boltzmann distribution, then the self-consistent gravitational-field approach implies using a configuration with infinite mass and infinite extent. This is obviously not applicable to real galaxies, and attempts to obtain a solution by cutting off the galaxy at a finite radius run into problems. The boundary conditions can dominate the form of the solution, which is not acceptable as a description of a distribution that appears remarkably uniform among galaxies in different environments.

A different approach is to find the most probable, or 'maximum-entropy', configuration of the system — taking into account the collisionless nature of the system. But this leads also to infinite extent, or to problems with singularities. The recent attempt by White and Narayan² to examine systematically the maximum-entropy states of gas spheres shows some interesting effects relevant to galaxies. If no constraints are put on the system other than finite size, mass and energy, and hydrostatic equilibrium, then the models of maximum entropy show that a very small portion of the mass is concentrated in a central 'spike', accounting for most of the binding energy of the galaxy, and most of the stars lie in an extended envelope. They liken the central singularity to Bose condensation in a quantum gas. It was already known that, under certain conditions, such a finite two-component structure can lead to a configuration of arbitrarily large entropy⁴. None of these two-component models has a density structure particularly good at reproducing that observed in real galaxies.

The difficulty could be that it is wrong to search for the global maximum of

entropy. All systems could finally evolve to a singular state over many times the present age of the Universe, but to explain the observed structure may only require a local maximum (or rather a slowly varying plateau) in entropy on the way. White and Narayan add² a further constraint to their model, that the density distribution must be a power law in radius. The exponent which gives the configuration with maximum entropy turns out to be the same as that found for real galaxies. This surely cannot be an accident; the global maximum for this artificially constrained system must represent some kind of local maximum for the real system.

What is still needed is an understanding of how the detailed mechanism of violent relaxation goes far enough to set up a well-defined energy distribution — possibly best described by a Boltzmann formula involving the binding energy of the stars⁵ — without going far enough to reach a global (but unrealistic) maximum-entropy state. This incomplete, but undoubtedly efficient, violent relaxation is clearly observed in numerical experiments⁶. To be effective, somewhat irregular initial conditions and a fairly large collapse factor (or some other large change in the overall gravitational field, such as accompanies a collision of two galaxies) are needed. Under these conditions the system presumably goes to its 'local' state of maximum entropy — in which the density falls off approximately as the inverse cube of the radius — and the collective violent relaxation dies out because the system stops changing shape and/or size long before the central condensation occurs. The initial and final energies of individual stars can still be somewhat correlated, again showing that the relaxation process is far from complete.

The importance of knowing the details of the violent relaxation process, and how far it can act before stopping, is acknowledged by all the leading proponents of the process^{2,3,7,8}. Now that extensive numerical simulations can be attempted guidance is badly needed. It would be valuable to find out what initial conditions lead to the observed structures of galaxies, even in very general terms of required collapse factor. The early history of the chemistry of, and star formation in, galaxies may not be intelligible without details of the dynamical processes. □

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Dutch association celebrates centennial

HET Nederlands Natuur – en Geneeskundig Congres (NNGC), the Dutch Association for the Advancement of Science, has just celebrated its 100th birthday with a jubilee meeting, "From Spark to Flame", held in Amsterdam, at which scientists from different fields reported on progress since the NNGC was first founded. The title of the conference refers to the biologist W.M. Beijerinck who, at the first meeting of the NNGC in 1887, spoke of Dutch science as "... a spark that he expected would develop into a flame during the years to come ...".

Despite recent severe governmental cuts in university spending, Dutch science is indeed flourishing 100 years later, both nationally and in international collabora-

researcher: a rather stubborn individual who likes to learn but tends to distrust the teaching of others. Conversely, he seldom has followers and does not form a school — for example, Van Leeuwenhoek, Stevin and Huygens, with Boerhave as the single exception. One of the original aims of the NNGC was to improve communication between scientists in Holland.

The NNGC held annual meetings that usually lasted 2 or 3 days. The fading photograph, taken in Leiden in 1907, shows that members of the governing boards included famous names such as Hugo de Vries, Lorentz, Zeeman, Eindhoven and Beijerinck. Members also included the mathematician L.E.J. Brouwer and E. Dubois, who in 1891 discovered



The 1907 annual meeting of the NNGC in Leiden. Seated: third from left, Lorentz (chairman), to the right of him Zeeman, then Eindhoven. First standing row, fourth from right, Hugo de Vries. Standing, third row from back on the far right, Beijerinck. Back row, far left, Kamerlingh Onnes.

tions. One area of interest is weak interactions and the top quark, investigated at CERN (Geneva) and DESY (Hamburg). In Utrecht, theoreticians are trying to see how close they can come to explaining gravitation without having to use the superstring theory. Dutch and British space scientists contribute to cosmic X-ray research by construction of a shadow mask camera. The detector consists of a position-sensitive proportional counter with a shadow mask in front. This camera will be incorporated in the Italian SAX satellite programme. A transmission-grating spectrometer is being developed for astronomical X-ray spectrometry that will be used in the US AXAF programme.

The NNGC was modelled after the Swiss *Société Helvétique* (founded in 1815), the German *Naturforscher Versammlung* (1822), the British Association (1831) and the *Association Française* (1871). The Dutch Association was founded late compared with those in other countries, probably because Holland is a small country that has always had strong international links: separate associations in each country were considered contrary to the universality of science. Despite these objections, the NNGC turned out to be a huge success.

The second reason for starting the NNGC was the specific character of the Dutch

Pithecanthropus Homo erectus alongside a river in Java. In 1907 Kamerlingh Onnes demonstrated his liquification of helium, proclaiming that he was well on the way to a temperature of absolute zero.

Right from the beginning the NNGC showed a democratic spirit. Although only academic people could become members, anyone interested in science could attend the annual meetings. The atmosphere at the congresses held at the turn of the century was marked by a mixture of solemnity and good nature. In 1927, when Eykman reported that his anti-beri-beri factor showed activity, the audience spontaneously broke out into applause.

The Second World War brought many changes. In 1943, by order of the Nazi occupation forces, Jewish names were removed from the list of members. Many NNGC members played an active role in the Dutch resistance. Since the war, the association's influence on Dutch science has waned as official Government agencies took its place in organizing and financing science. But the meetings of the association are still attended by enthusiastic audiences.

Mels Sluysen and Henny Daams

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Fish farming and influenza pandemics

Christoph Scholtissek and Ernest Naylor

Human influenza pandemics commonly arise by genetic reassortment between human and avian viruses in pigs. Yet global developments in aquaculture — the so-called 'Blue Revolution' — will mean increased co-location of people, ducks and pigs.

EVERY 10 to 20 years pandemic influenza A viruses with new surface antigens suddenly appear in nature, against which no neutralizing antibodies are present in the human population. This antigenic shift is caused by genetic reassortment; for example, in the pandemic Hong Kong virus of 1968 the haemagglutinin gene seems to have been contributed to by an avian influenza virus¹ which normally exists in a quite distinct reservoir of influenza A viruses in water fowl^{2,3}.

From experimental studies it seems that although human influenza viruses can multiply in ducks, they are not transmitted among these animals³. Presumably, too, avian influenza viruses are not transmitted among human beings, though (for obvious reasons) the experimental basis for that assumption has not been established. In fact the transmission of genetic material from avian to human influenza viruses appears to take place by reassortment in pigs³. There is firm evidence that pigs can become infected by and may transmit both human and avian influenza viruses not only amongst other pigs but also back to the original hosts⁴. Therefore, pigs seem to be 'mixing vessels' where the two separate reservoirs meet and where reassortment between avian and human influenza A viruses occurs, giving rise to the antigenic shift by creating new human pandemic influenza strains with new surface antigens.

Agricultural practices

Age-old agricultural practices in the region of South China, where pigs live in close contact with humans as well as with ducks, could facilitate reassortment of influenza viruses, which might explain why most pandemics start from that geographical area⁵. Yet, against this background, which suggests that it would be advisable to keep pigs as separate as possible from humans and from water fowl, the adoption of integrated farming and aquaculture systems involving livestock, fowl and fish is being actively encouraged.

The use of fresh manure from farm animals, particularly pigs and ducks, as fertilizer for fish ponds, has a long history in central Europe⁶ and also particularly in Asia⁷. Co-culture of fish and ducks, and of fish and pigs, is widespread in China^{8,9} and in Hong Kong, where both duck-fish and

pig-fish culture systems are scattered randomly throughout the main fish culture region⁷. Such polyculture systems are also common in India where, for example, the Central Inland Fisheries Research Institute (CIFRI) issues technical literature for small-scale fish farmers outlining procedures and costings for 'fish-cum-duck' and 'fish-cum-pig' culture. Such farmers in West Bengal are able to produce in fish-duck systems up to 4,000 kg ha⁻¹ yr⁻¹ and in fish-pig systems up to 7,000 kg ha⁻¹ yr⁻¹ of cultured carp with no supplementary feeding of the fish¹⁰.

More elaborate artisanal polyculture systems involving fish are also being developed rapidly in various parts of the world. In Thailand, for example, wide use is made of pig-hen-fish culture: the hens are in cages above the pigs which consume hen faeces, and the pigs are in pens directly above fish ponds into which they defecate⁷. In such systems both excess food from the farm animals and their faecal material are either consumed directly by fish or act as pond fertilizers. Similar systems involving ducks, pigs and fish are used in Hong Kong, Malaysia and Nepal⁷. Indeed, in Hong Kong, a geographical region from which it is known that influenza pandemics have arisen¹, fish culture activities are very extensive with 48.1 per cent devoted to duck-fish culture, 1.6 per cent to pig-fish culture and 12.2 per cent to duck-pig-fish culture⁷.

The rapid increase in human population in the developing world, where fish often provide a large component of the diet, has coincided with great pressures on wild-stock fisheries. Accordingly, the increased world demand for food has coincided with a major expansion in fish-farming, sometimes referred to as the 'Blue Revolution'¹¹. In the developing world there are obvious advantages in supporting fish-farming projects which are labour intensive and which at the same time provide low-cost fish feeds and pond fertilizers such as are available by combining aquaculture with agriculture. So it is not surprising that countries in the developing world, supported by organizations such as the Food and Agriculture Organization of the United Nations, the International Center for Living Aquatic Resource Management and the UK Overseas Development Administration, are all

recommending an increased use of artisanal systems of aquaculture integrated with farm livestock culture. Moreover, extension of such systems is being proposed not only in Asia but elsewhere, by transferring Asian techniques to rural development programmes in Africa⁸. Adoption of these recommendations will result in increased co-location of pigs, humans and ducks, concentrated in artisanal aquaculture industries, in a number of areas throughout the world consistent with religious considerations.

Health hazard

The result may well be creation of a considerable potential human health hazard by bringing together the two reservoirs of influenza A viruses, generating risks that have not hitherto been considered in assessment of the health constraints of integrated animal-fish farming⁷. In the context of a burgeoning aquaculture industry, they could have global implications for the recurrence of influenza pandemics. Review of integrated aquaculture systems, leading to the development of techniques which keep pigs in enclosed farms separate from waterfowl, is therefore a matter of some urgency. □

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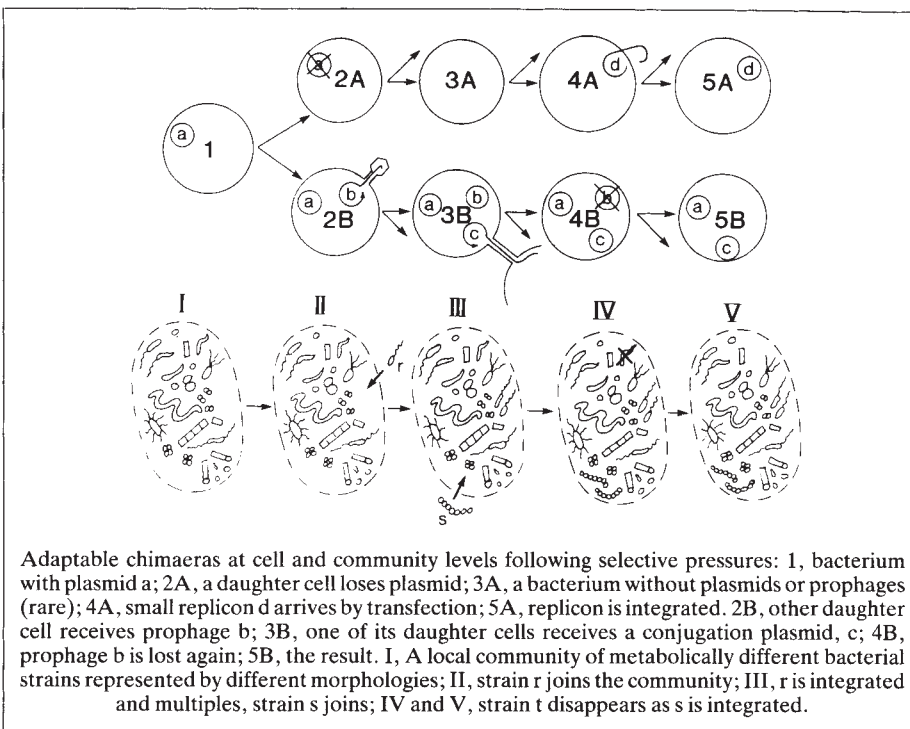
A bacterial way of life

SIR—A coherent concept of the bacterial world is long overdue. I feel this is largely because of the tendency to investigate bacterial strains out of the context in which they occur in nature. If, however, they are considered as adaptable chimaeras, a coherent concept emerges.

Bacteria live mostly in mixed local communities made up of metabolically different strains which support one another¹. Each community functions as a single organism, with an extremely efficient division of labour. Such integrated entities are found, for example, in soil and digestive tracts, and represent a bacterial solution to the need for a complex life strategy involving pluricellular participation. It is the equivalent of an individual plant or animal². New bacterial communities may be rapidly improvised by local selective pressures. They continually correct the mixture of their constituents by replacing less efficient strains with more appropriate ones. Therefore these dynamic communities — consisting of more than one genetic type of cells and able to switch their ecto-symbiotic strains — are adaptable chimaeras.

Similarly, at the cellular level, each bacterium is enriched by the temporary presence of one or more visiting genes, mostly small replicons — circular, self-replicating DNA molecules (prophages or plasmids). Their 'converting' genes encode potentially useful attributes, accessory to essential ones encoded by the large replicon ('chromosome' or nucleoid) of the cell. As soon as its usefulness disappears, a small replicon can be eliminated by 'curing', often to be replaced by a more appropriate one from the large pool that exists in many other strains. Specific receptors for such visiting genes are present at the surface of any bacterial cell. All categories of bacteria, including *Archaeobacteria*^{3,4}, possess at least one plasmid. Moreover, at least one in three strains contains one or more prophages (polylysogeny). Non-self-replicating DNA fragments may also be exchanged between different bacterial strains, although less frequently. When a favourable gene arrives in a cell from another strain, the newly formed cellular entity — also a temporary, adaptable chimaera — rapidly multiplies. When threatened by antibacterial substances, such as drugs² and active biological products used in agriculture, industrial or natural waste¹, many types of bacteria have survived by receiving resistance genes from soil communities.

Thus, as illustrated in the figure, there exists a general and efficient bacterial mechanism for solving problems and adapting both at the cellular and pluricellular levels. Genes and cells, most of which are unable to survive in isolation,



behave like easily associating basic components. Their potential is realized when they are combined into the best possible dynamic adaptable chimaeras. Only these are effective in nature and in step with permanent competition among bacteria. This general mechanism is suggestive of a computerized communication network². The information bank consists of all the bacterial genes. Selection proceeds through competition. Amplification results from a higher multiplication of the right combination. There are infinite natural circuits to facilitate the flow of genes or of cells. The chief role played by the bacterial world is based on such mechanisms. This simple generalized way of life enables bacteria to avoid extinction.

There is no close equivalence between the organization of eukaryotes and prokaryotes. The term chimaera, as used here, might be replaced by symbiosis⁵, or metabolic differentiation². The bacterial world seems to have evolved as a single loose and partially unified entity in which no gene or strain is entirely foreign. Therefore, there are no real bacterial species. The evolution of bacterial cells and small replicons available for the formation of efficient adaptable chimaeras took place for the possible benefit of the entire planetary bacterial world.

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Supernova γ -rays

SIR—A recent letter (Berezinsky, V.S. & Ginzburg, V.L. *Nature* **329**, 807–809; 1987) about the possibility of observing very-high-energy (VHE) γ -rays from the supernova SN1987A from the Southern Hemisphere pleaded for the rapid relocation of ground-based VHE γ -ray telescopes, and a news item (*Nature* **330**, 410; 1987) reported that two universities in Australia and New Zealand are racing to complete the construction of two such telescopes.

For the record, the University of Durham has been operating a large VHE γ -ray telescope in Narrabri, New South Wales (NSW), for more than a year. This telescope, on the site of the Sydney University cosmic-ray station at Narrabri, NSW, is part of a project, supported by the Science and Engineering Research Council, for γ -ray astronomy in the Southern and Northern Hemispheres and costing more than £500,000. The telescope is similar to, but about 50 per cent larger than, that being constructed by the University of Adelaide and comprises 141 mirrors, each of diameter 60 cm, in three groups. The light detectors at the prime foci are the most comprehensive yet used in VHE γ -ray astronomy and incorporate 21 photomultipliers giving new background control data particularly important for the supernova observations.

The Narrabri telescope has been operating on a planned programme of

observations of X-ray binaries and pulsars for more than a year, has accumulated about 1,000 hours of data, and has made many observations of the supernova at almost every opportunity since its discovery. Indeed the telescope was making measurements of the pulsar PSR0540, at the position of the supernova, in February 1987. (One observing run started just a few hours after the neutrino burst.)

Observations on several days before and after the core collapse indicate a limit on the continuous emission of 250-GeV γ -rays of 10^{-10} photons $\text{cm}^{-2} \text{s}^{-1}$ immediately following the supernova. This result is not surprising, as it is uncertain within a year or so when, how quickly and, indeed, if the supernova will become a source of VHE γ -rays. Other results taken in September 1987 indicate a similar flux limit.

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How to avoid jet lag

SIR—In a News and Views article¹, Winfree asks whether the observation of Mrosovsky and Salmon² — that hamsters adjust much more quickly to a large change in the light-darkness cycle if, when this change is made, the animal is put on a running wheel so that it will be active at a time when, before the cycle change, it was asleep — is relevant to the problem of minimizing jet lag in people.

In my experience, after flights of 12 hours or so associated with a large change in longitude, one recovers much more quickly if the flight ends in the late afternoon or evening, as may be the case when travelling from London to Singapore or Los Angeles, than when one arrives in the early morning, as seems almost inevitable in London. In the former case one has dinner after arrival at about the time when, before the journey, one would probably still be asleep (although the local clock agrees that it is dinner time) and then goes to bed. In the latter case one either goes to bed when the local clock says that it is time to get up, or goes to work while suffering from lack of sleep.

This anecdotal evidence is purely subjective, and uncontrolled variables such as the direction in which one was travelling and how well one slept on the aircraft may affect the situation. It might be rewarding, however, to conduct some experiments in which these variables were controlled and subjective feelings were supplemented by objective tests of recovery from jet lag.

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Joints of the crocodile-reversed archosaurs

SIR—Cruickshank and Benton¹ challenged the hypothesis of monophyly in the so-called crocodile-reversed archosaurs (Euparkeriidae and Ornithosuchidae). Their arguments hinged on the idea that a unique derived tarsal structure is not synapomorphic for the two families and questioned the concept of two divergent early archosaur lineages²⁻⁷. Here we present synapomorphies of the tarsi of *Euparkeria* and ornithosuchids as evidence of the monophyly of the crocodile-reversed lineage.

In crocodile-reversed archosaurs, the proximal of two primitive intratarsal facet pairs became the principal ankle joint^{2,7,8}. The mobile astragalocalcaneal joint in *Euparkeria* is a cylinder-in-cylinder, whereas it is a true ball-in-socket in the ornithosuchid *Riojasuchus*. The rotational axis of the calcaneum on the astragalus (MAJ) is oblique to the long axis between crus and tarsus (UAJ) in *Euparkeria*, whereas the two axes are parallel in *Riojasuchus* (Fig. 1). The calcaneal facet on the astragalus faces ventrolaterally, is oriented horizontally, and terminates medially in the main concavity for calcaneal articulation. Its location is ventrolateral, not distal, in both *Euparkeria* and *Riojasuchus*. The facet is an anteroposteriorly elongate groove in *Euparkeria*, whereas it is medio-laterally aligned and terminates medially in a conical depression in *Riojasuchus*.

The calcaneal facets for astragalus articulation are similar in structure in *Euparkeria* and *Riojasuchus*. A surface shaped like a partial cylinder terminates medially in a conical eminence. The two genera differ in that the conical eminence

of the astragalus facet in *Riojasuchus* fits into the conical concavity on the ventrolateral astragalus, whereas the entire articulation in *Euparkeria* is by the hemicylindrical convexity on the anteromedial calcaneum fitting into the cylindrical concavity on the astragalus. The structure of the astragalus facet and the medially recurved distal end of the calcaneal tuber lacking a medial groove are derived characters shared only by *Euparkeria* and the ornithosuchids⁷.

Cruickshank and Benton stated¹ that the tuber calcis of *Euparkeria* is lateral in orientation and "of the same basic pattern as in *Proterosuchus* and similar forms". Relative to the long axis between crus and proximal tarsus (UAJ) in *Euparkeria*, the tuber long axis (TLA) projects posterolaterally, at an angle of 25°, slightly less than in living crocodilians (30°–58°). A posterolateral tuber allows flexion of the gastrocnemius to be transmitted to a laterally oriented pes in sprawling and 'semi-erect' archosaurs (for example, phytosaurs and crocodilians)⁷. A truly lateral tuber is only seen in early diapsids (for instance, *Proterosuchus*) with an immobile MAJ. A posteriorly directed tuber of the type seen in *Riojasuchus* is seen elsewhere only in archosaurs with parasagittal limb mechanics (for example, aetosaurs and poposaurids)^{6,7}. Reorientation of the TLA, UAJ and MAJ can be linked to a shift from sprawling to erect posture⁷ along a gradient consisting of *Proterosuchus*, *Euparkeria* and *Riojasuchus*.

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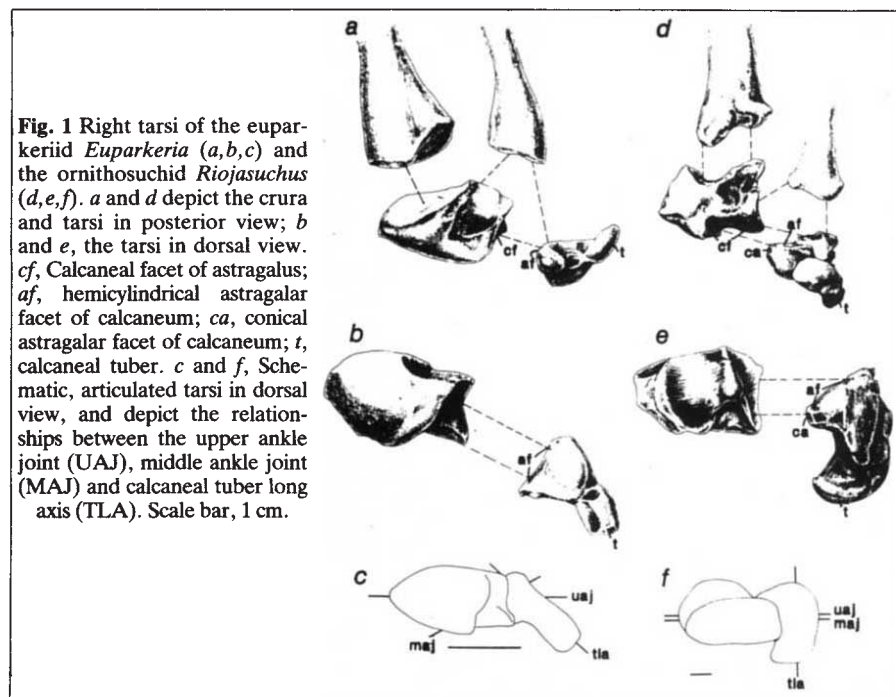


Fig. 1 Right tarsi of the euparkeriid *Euparkeria* (a,b,c) and the ornithosuchid *Riojasuchus* (d,e,f). a and d depict the crura and tarsi in posterior view; b and e, the tarsi in dorsal view. cf, Calcaneal facet of astragalus; af, hemicylindrical astragalus facet of calcaneum; ca, conical astragalus facet of calcaneum; t, calcaneal tuber. c and f, Schematic, articulated tarsi in dorsal view, and depict the relationships between the upper ankle joint (UAJ), middle ankle joint (MAJ) and calcaneal tuber long axis (TLA). Scale bar, 1 cm.

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SIR—Cruickshank and Benton¹ reinterpreted the ankle joint of the early archosaur *Euparkeria*, which they described as having a modified primitive mesotarsal (MPM) joint, rather than the crocodile reversed (CR) joint usually attributed to that taxon^{2–5}. Three other ankle joints are recognized in archosaurs: the ancestral primitive mesotarsal (PM) joint, crocodile normal (CN) and advanced mesotarsal (AM) joints. The evolutionary history of these joints is controversial^{1–5}. Cruickshank and Benton did not escape two conceptual errors: (1) they treated homology as if it were an inductive rather than a deductive concept; and (2) they invoked hypothesized evolutionary processes to support their preferred phylogenetic pattern, instead of using the less theory-laden pattern to test hypotheses about evolutionary processes. For the purposes of this argument, the proposition that archosaurs have five different and non-overlapping types of ankle joint is accepted, although this does not appear to be the case⁴.

The first problem stems from Cruickshank and Benton's¹ assertion that differences between the ankle joints of *Euparkeria* and Ornithosuchidae (CR) preclude homology. To be homologous, two characters need not be particularly similar, but must derive from a common ancestor⁶. For example, the form and function of the quadrate bone in living mammals is radically different from that of their extinct relatives, but the elements are homologous⁷. Cruickshank and Benton¹ admitted that the peg-in-socket ankle joints of *Euparkeria* and Ornithosuchidae share a derived condition in that the peg is on the calcaneum and the socket is in the astragalus. The shared derived characters that are consistent with the preferred phylogeny are interpreted as homologous, whereas those that are discordant with it are homoplastic⁸. Thus, if *Euparkeria* and Ornithosuchidae are closely related, the derived resemblance would be most simply explained as homologous, even if their ankle joints are otherwise completely different. Conversely, if they are not, then their ankle joints would be homoplastic, no matter how similar they might be. So the problem reduces to the relationships of *Euparkeria*: is it the sister group of other archosaurs as Benton suggested⁹, or is it on the ornithosuchian side of the archosaur tree as others have argued^{2–5}?

The second problem faces all phylo-

genetic analyses of archosaur ankle joints. The problem stems from assumptions about evolutionary processes underlying the ordering of states in a multistate character, which can be illustrated as follows. Characters having two states, an ancestral condition (0) and one derived therefrom (1), are termed binary characters and minimally they imply one evolutionary transformation and specify a single character tree. But even the minimum number of possible character trees rises dramatically with an increasing number of states. For example, a three-state character (two transformations) yields three equally parsimonious character trees [(0–1–2), (0–2–1), or (0–1 and 0–2)], and a four-state character (three transformations) yields 16, and so on. Given that the archosaur ankle joint has five states — 0(PM), 1(MPM), 2(CN), 3(CR) and 4(AM) — numerous character trees, all with a minimum of four transformations, are possible. An unambiguous basis¹⁰ for choosing one among these alternatives requires addition of more taxa and binary characters. That is, to conclude that state 0 transformed into state 1 and then into 2, for example, there must be at least four taxa (= 2 × number of transformations) and two binary characters (= 2 × number of transformations – 2), arrayed in the phylogeny (0(1(1 2))). Thus, demonstrating a linear evolutionary sequence among archosaur ankle joints requires a minimum of eight taxa and six binary characters. This reduces to a problem of evolutionary rates; characters that evolve less rapidly (binary) provide a clearer picture of phylogeny than do characters that evolve more rapidly (multistate). An archosaur phylogeny derived from binary characters will probably tell us more about the evolution of archosaur ankle joints than the ankle joints will be able to tell us about archosaur phylogeny.

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CRUICKSHANK AND BENTON REPLY—We believe the following may be the points of contention: (1) the nature of the ankle in *Euparkeria*; (2) the question of homology; and (3) the need to examine as many parts of an animal as possible before coming to decisions about relationships. Above, Parrish illustrates a right ankle of *Euparkeria* but gives no provenance for it. These

tarsal bones differ in detail from those illustrated elsewhere¹ and the reconstructions are also different. The bones of the ankle in *Euparkeria* are very small, but seem to be so close to those of *Chanaresuchus* and in some important respects to that of *Erythrosuchus*, representatives of thecodontian families which range into the Upper Triassic, that we have to regard them as an independent radiation of the Archosauria.

It would be a neat solution to be able to continue to support the contention that there was an evolutionary gradient leading from *Proterosuchus* through *Euparkeria* to *Riojasuchus*, but if we are to restrict the argument entirely to ankle morphology, then the problem centres on *Euparkeria*. We have difficulty in correlating the orientation and reconstructed relationships of the *Euparkeria* ankle bones illustrated by Parrish with those illustrated elsewhere^{1,2}. The essential feature of the calcaneum is the retention of a deep, posteriorly directed pyramid, which articulated with the complementary recess in the astragalus. This would allow restricted anteroposterior movement only. This is anatomically and functionally so far removed from the condition in *Riojasuchus* that we still have great difficulty reconciling the homologies of the two forms. *Euparkeria* and *Riojasuchus* may be said to have similar astragalo-calcaneal articulations — structures shaped like partial cylinders fitting into recesses on the distal faces of astragali — but their axes of rotation are at right angles to one another by our interpretation.

Gauthier argues that we have failed to understand the concept of homology. This is a larger question than can be properly answered here, and is not entirely relevant to the original paper³. We argued that the ankles of *Euparkeria* and *Riojasuchus* are not homologous on the basis of detailed anatomy, and not on the basis of an assumed pattern of evolution. We agree with Gauthier that a phylogeny of archosaurs must be based on an analysis of as many characters as possible, from all parts of the skull and skeleton³. Our paper was intended as a commentary on the large numbers of papers that had been published recently on archosaur ankles, not as a final phylogenetic statement.

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Using common sense

Colin McGinn

Psychosemantics: The Problem of Meaning in the Philosophy of Mind. By Jerry A. Fodor. MIT Press:1987. Pp.171. \$20, £17.95.

WHAT is the scientific status of common-sense psychology? Should it be taken as a sound basis from which to build a psychological science — needing to be deepened and extended, certainly, but right in essentials? Or should it be discarded wholesale as so much outworn superstition, fit only to be replaced by some quite new kind of theoretical structure? The latter attitude prevailed during much of the infancy and adolescence of scientific psychology, but the former attitude seems to be gaining ground in the (relatively) mature period we know as cognitive science.

Jerry Fodor is a firm conservationist: he thinks that modern cognitive psychology vindicates the constructs of ordinary belief-desire explanation — in particular, he thinks that the idea of mental representation is common to both and is a fine thing in itself. His aim in this book is to protect folk psychology, as a solid basis for mental science, from a range of objections that have been brought against it in recent years, mainly by philosophers. He does so with verve, clarity and wit, generally getting the better of his revisionary opponents. The book is vintage Fodor: clever, stimulating, challenging, infuriating. It will undoubtedly become the target of much critical discussion as the philosophy of psychology moves towards its adulthood.

According to Fodor, folk psychology works wonderfully in ordinary life and is practically indispensable. Its predictive power is no accident, he claims, because it is a *deep* theory, in the way that physics is deep. The depth comes from two features of the theory: the fact that it postulates unobservables, and its preference for causal intricacy over proliferation of theoretical primitives. This is a neat point against old-style positivistic behaviourists, making unobservability a virtue rather than a liability; their mistake, ironically, was to fail to take the model of physics seriously enough. But Fodor neglects to mention an equally salient feature of folk psychology: the fact that it postulates introspectables — for we also have direct first-person access to our beliefs and desires. By Fodor's criterion of depth, this feature should make us say that folk psychology is *superficial*. Here is where it differs from physics. What would he say about this difference?

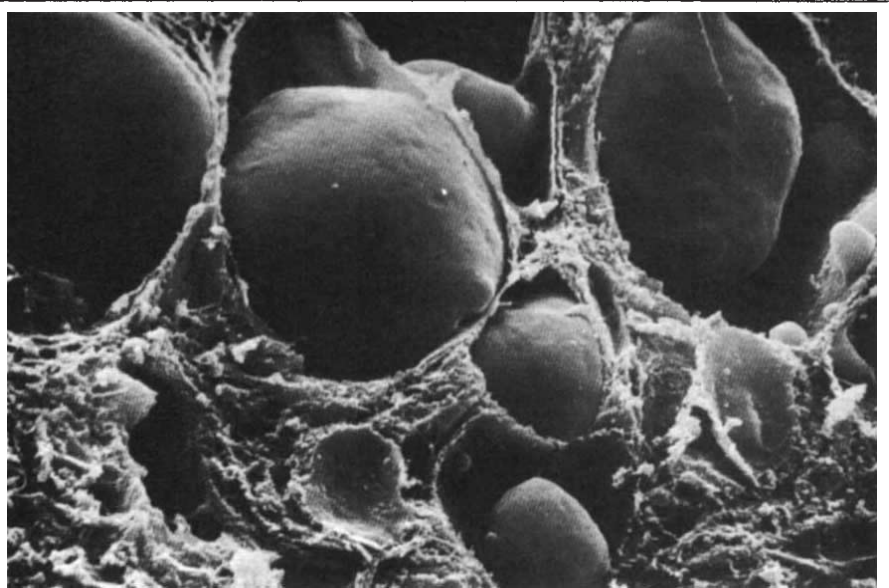
The scientific theory that vindicates belief-desire psychology, Fodor assures

us, is the computational conception of mental processes. This kind of psychology treats the mind as a symbol-manipulating system, the mental symbols having both causal and semantic properties. Just as we commonly suppose a rational harmony between what a belief logically implies and what it is disposed to cause, so the idea of a language of thought integrates syntactic shape and semantic content — thus explaining how a rational mechanism might be constructed. This may be, as Fodor says, "a perfectly terrific idea", but it is not — contrary to his repeated assertions — the only idea in the field. Psychologists (such as Philip Johnson-Laird) who frame their theories in terms of mental models will be surprised to find their approach declared to be non-existent. In general, Fodor is far too ready to move from the need for structured mental representations to a specifically sentential conception of the form of these representations. In arguing for the language of thought, indeed, he focuses on an example of a mental representation, namely a tree-structure representing an understood sentence, that is in fact more a model of a sentence than a verbal description of one — supposing this to support his specifically linguistic theory of mental representation.

With folk psychology shown to be, on the face of it, in good theoretical standing, Fodor goes on to rebut three potential threats to its security: externalism, holism, naturalism. Externalism claims that the content of a belief can be pulled apart from its causal powers, so that any science geared to capturing causal generalizations will have no use for the ordinary notion of content. Fodor accepts this argument, stating it with considerable force, but evades its alleged conclusion by confecting a notion of content ("narrow content") that cannot be divorced from causal powers. Supervenience on the physical is thus respected, but at the price of ineffability in the kind of content that so supervenes.

I think Fodor is right to discern this kind of content, but he underestimates the magnitude of the concession he makes by banning wide content from psychology. For, by his own showing, narrow content cannot be (exclusively) specified in the terms of folk psychology: so a psychology based on narrow content will be neither expressible nor a version of ordinary folk psychology; it will not have available the form 'x believes that p'. The lesson is to look more critically at the original demand to make psychology the study of the causal powers of mental states.

Holism takes the content of a belief to be fixed by the totality of beliefs with which the given one has 'epistemic liaisons'. It thus blocks generalizing over believers, since believers will always differ in their total belief-sets. Fodor convincingly demolishes a number of arguments for this extreme holistic thesis, and opts for a local denotational theory of content.



First among equals — only one of about 20 follicles produced each month in the cortex of the human ovary develops to the point where it produces a mature egg. This scanning electron micrograph (×1211) is taken from Microcosmos edited by J. Burgess, M. Marten & R. Taylor, a collection of micrographs from science and technology. The book is published by Cambridge University Press, price £15, \$24.95.

He also rejects theories that regard content as the fusion of internal and external factors, though far less convincingly. First, he mistakenly assumes that 'two-factor' theories take each factor to determine a unique proposition: but the whole point of such theories is that this is not so. Second, his own earlier notion of narrow content supplies precisely what the two-factor theorist needs to rebut Fodor's criticism. Third, Fodor's reluctance to allow any place for functional role in the fixation of content sits ill with his previous claim that content is conferred by a harmony between inferential propensities and logical consequence.

Naturalism would be a threat if we could not explain mental reference in naturalistic terms. Fodor tries to develop a causal covariation theory of reference, thus explaining where meaning fits in the natural order. This is an ingenious discussion, but problems bristle — in particular, the problem of explaining the possession of content in the absence of appropriate environmental entities. What would he say about the brain in a vat? It looks as if he has to say, implausibly, that the causally isolated terms in its language of thought either have no content or some very bizarre sort of content concerning nerve-endings or some such. I think Fodor should reconsider the prospects for a teleological theory, which he dismisses too quickly. Pure causal theories face formidable problems, especially with respect to the phenomenological content of perceptual experience — a type of content he conspicuously fails to discuss.

Fodor may not have the last word on all issues, as he would be the first to admit. But his forthrightness and intellectual daring are the best way to push our understanding forward. *Psychosemantics* is a notable contribution to the old question of how the mind represents the world. □

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• Daniel Dennett is another philosopher who believes in the value of commonsense psychology. But in *The Intentional Stance*, a collection of his recent essays also published by MIT Press (\$25, £19.95), he parts company with Fodor by arguing that the pattern of commonsense explanation is not necessarily mirrored in a "language of thought" in the brain. Connectionist models might just turn out to provide one alternative, according to Dennett, for neural networks appear to perform cognitive tasks — the correct pronunciation of written English, for example — without any rules being explicitly represented anywhere in the network.

Dennett's "intentional stance" leads him through a rich and varied landscape, from speculations on frog psychology to a critique of adaptionism that should do

much to silence those biologists who believe that philosophers never have anything useful to say to them.

An encounter with ethologists provokes two of the most entertaining essays. Dennett sets off for Kenya, with primatologist Robert Seyfarth, to see (tentatively) if light can be shed on the remarkable communication abilities of vervet monkeys by adopting the intentional stance; that is, by using explanatory intentional idioms, such as 'believes' or 'wants'. The result is surprising: Dennett, retaining philosophical rigour, is able to develop indirectly testable hypotheses of what particular monkey calls might mean. But his method leads him also to conclude that vervet monkeys "like honeymooners who have not been out of each other's sight for days" have rather little to say to one another. For a more complex language to be useful, a few more opportunities for deceit are required!

Alun Anderson

Fighting fungi

G.A. Pepin

Introduction to the History of Medical and Veterinary Mycology. By G.C. Ainsworth. Cambridge University Press: 1987. Pp.228. £30, \$54.50.

IN ITS popular image, mycology deals with mushrooms, moulds and mildew. This view may extend to the wider range of fungal infections in plants but usually stops short of human or animal disease. Yet, world-wide, millions of people and their livestock are infected by fungi. Many of these infections cause only mild disease but others do not: they disfigure, disable or kill. Moreover, fungal growth in food crops, before or after harvest, sometimes produces potent toxins that cause disease if eaten, and millions of dollars have been spent over recent years in efforts to prevent them entering the diet of animals or human beings.

Despite its title, Dr Ainsworth's book is not solely for historians; anyone interested in the biology of infectious disease should find it stimulating, entertaining and instructive. He traces knowledge of diseases caused by fungi from antiquity to the present day, but points out that by far the greatest progress in medical and veterinary mycology occurred only within the past 50 years. Why did such problems attract so little effective attention for so long? After all, fungal infections were the first to be scientifically proven in the nineteenth century, when the whole notion of disease caused by microbial invasion was new and controversial.

Segregation within science was partly to blame. Once the relative importance of bacteria and, later, viruses in the epi-

demic, contagious diseases became apparent, medical attention was focused upon them, and the new sciences of bacteriology and virology developed in close alliance with clinical research. Mycology, on the other hand, remained within the province of botanists, whose interests and experience led them to concentrate upon fungi responsible for plant diseases. Out of their work grew a body of knowledge, methods and skills relevant to medical mycology but rarely applied to it until the 1930s, when the need



St Antony with a victim of gangrenous ergotism, which can be contracted from the rye used for baking bread (H. von Gersdorff, *Feldbuch der Wundartzney*, 1551). Courtesy of Geoffrey C. Ainsworth.

for clinicians and mycologists to collaborate at last began to be accepted.

Throughout his career Dr Ainsworth has been active in furthering such collaboration and the consequent advance of medical and veterinary mycology. With a wide knowledge of mycology, its applications and history, coupled with a talent for conveying lucidly and succinctly the essence of his subject, he is uniquely able to communicate both the difficulties and the achievements of workers in this field.

More than a history, this short, scholarly book is an up-to-date synopsis of the subject, summarizing both past and present approaches to most of its essential problems and briefly reviewing progress in the various regions of the world. Particularly valuable is the extensive and well-organized bibliography. There the reader can easily find, directly or by way of an index, further sources or references pertinent to any topic mentioned in the text. □

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NMR bagatelle

K. A. McLauchlan

Nuclear Magnetic Resonance: General Concepts and Applications. By William W. Paudler. Wiley: 1987. Pp.291. £32.10, \$35.

Introduction to Multinuclear NMR: Theory and Application. By Claude H. Yoder and Charles D. Schaeffer, Jr. Benjamin/Cummings: 1987. Pp.335. Pbk \$27.95, £16.95.

Modern NMR Spectroscopy: A Guide for Chemists. By Jeremy K.M. Sanders and Brian K. Hunter. Oxford University Press: 1987. Pp.308. Hbk £35, \$60; pbk £17, \$35.

Multinuclear NMR. Edited by Joan Mason. Plenum: 1987. Pp.639. \$115 (North America); \$138 (elsewhere).

A Handbook of Nuclear Magnetic Resonance. By Ray Freeman. Longman, Harlow, UK/Wiley, New York: 1987. Pp.312. Hbk £18.95, \$49.95; pbk £14.95.

Principles of Nuclear Magnetic Resonance in One and Two Dimensions. By Richard R. Ernst, Geoffrey Bodenhausen and Alexander Wokaun. Clarendon: 1987. Pp.610. £60, \$98.

THE impact of nuclear magnetic resonance on chemistry is inestimable and its influence in biochemistry and medicine continually increases. Its applications have always been led by its methodology and techniques, transformed in recent years by pulse experiments of increasing sophistication. Among the most influential figures in its development have been Richard Ernst and Ray Freeman, whose contributions have affected the whole development of molecular science and who appear as a co-author and author, respectively, of two of the volumes under review. The breadth of the subject requires books to explain the basic ideas and uses, to describe applications, to introduce the chemist to the latest methods, to give the experimentalist real insight and to provide the detailed theoretical justification for everything. All are represented here.

Paudler's introductory volume considers many nuclei and provides simple problems for the reader. The wide coverage has resulted in the detail provided on any one topic being too small to be useful; for example, the spin-spin coupling between 11 pairs of nuclei are discussed in 19 pages. The approach is didactic, few pages are devoted to the modern methods, references are largely from the 1960-1970 era and the book has an old-fashioned air.

Yoder and Schaeffer are experienced teachers who provide a good first introduction to multinuclear NMR. Theirs is a no-nonsense practical approach, stronger

on chemistry and applications than on the physical basis. The reader gains a clear idea of what NMR can do, but I particularly liked the 80 pages devoted to its interdependence with other physical methods for structural analysis. Little space is devoted to multi-pulse and two-dimensional methods, but the clear exposition of basic ideas and applications makes this a good undergraduate text.

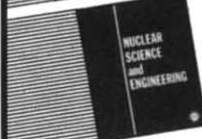
The application of modern methods to chemistry is the exclusive subject considered by Sanders and Hunter. The approach is non-mathematical to the extent that although the results of Fourier transformation pervade the book the transform itself goes undefined. All of the main modern techniques receive attention and readers are instructed in what these techniques can tell them and in how to go about the experiments and the analysis of results. The emphasis is on problem-solving and I cannot imagine anyone using NMR in practical chemistry failing to find this book useful. It was good to see a chapter on solids and one which exemplifies what can be achieved by applying several techniques to one molecule, a novel and welcome feature.

The volume edited by Mason contains 23 chapters from 12 authors and is overwhelmingly for the inorganic chemist wishing to apply NMR techniques to any magnetic nucleus; a brief chapter on biomedical applications seems out of place. This is a book for the research user, although introductory chapters describe the basis of the subject and the interpretation of its derived parameters. Each contribution is well referenced, although some are more up to date than others. With such a general coverage it is perhaps inevitable that the chapters are mainly descriptive, and they largely lack critical bite. However the book will prove useful — though not indispensable — to those it is aimed at.

Freeman, predictably, caters for practising NMR scientists who want a detailed understanding of the techniques they use; applications have no part. His book is of unusual form, constituting an alphabet of NMR with 59 entries on different aspects, ranging from A for 'apodization' to Z for 'zero-filling'. Each entry is redolent with flair and insight, and the author's achievement has been to be correct whilst being brief. He has an unerring ability to go to the heart of a problem and illuminate it. Admirers of his lectures will be pleased to learn that the book is full of his famous cartoons. It is a delight, and a necessary part of any serious NMR book collection.

The requirement for a major monograph on the subject of pulse NMR spectroscopy in one and two dimensions has been met by Ernst, Bodenhausen and Wokaun. The book is a triumph of careful and clear exposition, ranging over liquids, solids and spin-imaging, with all the new

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methods subject to detailed examination and illustrated with examples. It is scholarly and readable, with the exception of an idiosyncratic first chapter full of ponderous humour which the authors may already regret. This will be the lasting text of its age, the Abridgement amongst modern NMR books.

The last two books will be invaluable to the NMR scientist. Of the others, three deserve consideration in their various ways, but whether they are better than existing texts will be a matter of personal prejudice. Those by A.E. Derome (*Modern NMR Techniques for Chemistry Research*; Pergamon, 1987) and by R.K. Harris (*NMR Spectroscopy*; Pitman, 1983) are certainly strong competitors. □

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• A book that arrived in the Nature office after this review is *Two-Dimensional NMR Spectroscopy: Applications For Chemists and Biochemists* edited by William R. Croasmun and Robert M.K. Carlson. The book, just published by VCH, is part of the Methods in Stereochemical Analysis series and is aimed at chemists and biochemists who are not primarily NMR spectroscopists, but who may wish to apply the new techniques of two-dimensional NMR to their fields of interest, along with researchers who already use these techniques, but not yet in two dimensions. Price is DM210, \$104.50.

Maintaining the balance

John Maddox

Strategic Defenses and Arms Control. Edited by Alvin M. Weinberg and Jack N. Barkenbus. Paragon House, New York: 1987. Pp.263. \$24.95.

MARCH 23 1983, when President Ronald Reagan made public his dream of constructing a comprehensive defence against ballistic missiles, known as the Strategic Defense Initiative (SDI), was a fateful day for would-be arms controllers and their well-wishers. After a quarter of a century of labour in the negotiating rooms, they could boast of only two substantial agreements that enjoyed the benefit of having been ratified — the partial test-ban treaty of 1963 and the Anti-Ballistic Missile (ABM) treaty, ratified in 1972. The offer of SDI inevitably, therefore, gave offence to those who listened carefully five years ago, not merely because it seemed like making science fiction the cornerstone of policy but because success would spell the end of the ABM treaty.

Alvin Weinberg is one of the world's reasonable men (which is not yet a pejorative term). He and his co-editor, Jack Barkenbus, have drawn together a symposium of the views of people who do not instinctively cry "foul" at the suggestion that strategic defence may have a useful part to play, not merely in military security, but in the process of arms control itself. Their book is neither a case for building comprehensive defences against ballistic missiles nor an evaluation of SDI as such. Rather, it is an examination of the ways in which strategic defences could contribute to stability and even arms control. That by doing so they will offend some of those to whom the ABM treaty has become a shibboleth is neither here nor there, and certainly not their fault.

Nevertheless, it would have helped the general understanding — and the general case that Weinberg and Barkenbus seek to make — if they had more explicitly reminded their readers of the great arguments about the ABM treaty in the four years preceding its ratification. There is no echo here of the passionate intellectual clashes in the United States between members of the President's Science Advisory Committee (not yet abolished) and of the US Congress on the one hand, and the military on the other.

By what magic, in the end, did the ABM treaty come to be agreed? In Washington, the telling case was the wish to protect the doctrine of mutually assured destruction (leading to deterrence) from erosion by effective defensive systems, which was probably also the clinching argument

in Brezhnev's Soviet Union. But liberal opinion in the United States paid more attention to the prospect that a competition to build ground-based anti-missile missiles would be ruinously expensive, and probably ineffectual as well. The Pentagon, chastened by the problems of making the Sprint missile function properly, had little choice but acquiescence.

What has changed? Weinberg and Barkenbus say they are disappointed that the ABM treaty did not persuade the superpowers to stand still in the accumulation of offensive weapons, but was that ever on the cards? That, surely, was the function of the successful negotiation of the limitations of strategic arms in the 1970s (when, the editors say, arms control was given undue attention). Even now, the new treaty on Euromissiles notwithstanding, these agreements remain worthwhile. Meanwhile, the old arguments in favour of the ABM treaty live on in the arguments against SDI.

Yet Weinberg and Barkenbus are right to have raised their awkward question.

SDI in the sense of President Reagan's vision will probably not long outlast his presidency, but it would be folly to believe it will leave no mark on the strategic relationship between the United States and the Soviet Union. What, for example, will be the role of infrared satellites for early warning of missile attack in future years?

What Weinberg and Barkenbus plead for, in their concluding statement, is a managed transition to a "defense dominated world", in which for example the protection of missile sites will be given priority over the protection of civilian populations (so as not to destabilize deterrence). Nobody will dispute their intentions, but only the adequacy of the fund of rationality necessary to effect such a subtle transition. May it not be a better to hope that the arms control process will in due course serve what has always been a large part of its purpose — to encourage a political climate in which the dangers of conflict are intrinsically reduced?

John Maddox is editor of *Nature*

Flower power

Keith Allen

The Origins of Angiosperms and Their Biological Consequences. Edited by Else Marie Friis, William G. Chaloner and Peter R. Crane. Cambridge University Press: 1987. Pp.358. Hbk £27.50, \$44.50; pbk £9.95, \$16.95.

AN increasing number of scientific books are edited compilations of papers. They loosely fall into three categories: those resulting from a general conference or symposium; those based on a more specific scientific meeting; and those in which the editor(s) invite selected authorities to cover what they feel are the most important topics for a well-balanced volume. In general, books in the first category are the least satisfactory, comprising a *mélange* of papers (chapters) which often have little relationship to one another, or to an overall theme. The best are usually those in which the editors are given a free hand in selecting topics.

The Origins of Angiosperms and Their Biological Consequences is the result mostly of papers presented at a conference of the same name and happily has the unity of an 'editor-selected' book. On reading the title and before seeing the chapters, I wrote a list of the topics I would expect to be covered. Almost all are present, although I had listed a chapter on fossil soils with reference to angiosperm, metazoan and fungal interrelationships, and I expected more discussion of afforestation.

The editors are to be congratulated on a

number of counts. They have written a useful introduction which is followed by a good sequencing of chapters. They have confined the less readable statistical information to appendices and they conclude with a glossary of terms and the classification used throughout the book. (I note, however, that P. Crane uses the word Pteridophytes to include both the LycopHYta and Pteridophyta.)

The ten chapters are well written and understandable. They cover the origins of angiosperms relating the groups through cladistic analysis, changes in vegetation resulting from angiosperm diversification and various aspects of plant/animal interactions and interrelationships. The palaeogeography and palaeoclimatology at the time of early angiosperm evolution is discussed. One small criticism is that the last chapter, although well written and informative, is more provincial in content when compared with the more general theme of the earlier chapters.

Within university teaching, my impression is that during the last five to ten years there has been a move away from general taxonomy towards a study of the biologically related aspects of both fossil and recent plants and animals. Palaeoecosystems and coevolution, for example, can fire the imagination of the student, and this book fills an important gap in the literature.

Its interdisciplinary approach should attract a wide audience in both biological and earth sciences. It should be widely read, but I expect it to be of especial interest to the third-year undergraduate. □

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The ribosome returns

Peter B. Moore

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Fashions come and go in biochemistry. The discovery that some RNAs are enzymes is reviving interest in the long-neglected ribosome.

BIOCHEMISTRY is a science whose innumerable specialities are of unequal status. There are, of course, differences of opinion about what the pecking order of its subdisciplines is, or should be, with the happy result that not everyone works on the same problems at the same time. (It only seems that way.) Furthermore, rank order changes. An area which is modish one year may be *passé* the next, and recovery of status is possible too. Indeed the purpose of this piece is to announce the resurrection of ribosome biochemistry (*floruit*: 1955–1965).

The Golden Age

The Cold Spring Harbor Symposium of 1969 was entitled "The Mechanism of Protein Synthesis", and its proceedings¹ are a comprehensive statement of what was known about the translational aspects of gene expression at the close of the classical era of molecular biology. About half the papers presented dealt with ribosome structure and function, eloquent testimony that ribosome biochemistry was respectable in the late 1960s.

By 1969, it had been established that the ribosome is a polymerase that uses aminoacyl transfer RNAs as substrates for making protein under the direction of messenger RNA templates. The ribosome is the largest of the polymerases: its relative molecular mass (M_r) is about 2.5×10^6 in *Escherichia coli*, the organism whose ribosomes were, and still are, best understood. Ribosomes have two subunits that have independent roles in the initiation of protein synthesis, but work together to catalyse peptide bond formation; the duty cycle of the ribosome was well understood (Fig. 1). What distinguishes the ribosome decisively from all other polymerases are the three RNA molecules in its subunits, which together are responsible for over half its mass. The rest is accounted for by ~50 different proteins of low molecular mass, each present in one or a few copies per particle. (The details of its composition, depicted in Fig. 4, were not fully understood until a few years after 1969.) The physical basis for ribosome biogenesis was also clear: the 22 components of the small ribosomal subunit reconstitute *in vitro*.

Decline and fall

There are many reasons why ribosome research fell out of favour in the years that followed. First, the questions had changed. It was no longer what ribosomes do, but how they do it that was interesting, and mechanistic questions are not everyone's cup of tea. Second, ribosomes appeared to be unique; no other RNA-containing enzymes were known. Are ribosomes ordinary protein enzymes accidentally trapped in a matrix of RNA, or are they RNA enzymes stabilized by protein? Some people were uncomfortable dealing with a molecular freak; others found heresy unattractive. Third, the ribosome looked intractable. Its RNA was too large to work on using the technology of 1970. Its proteins are relatively insoluble and lack assayable activities. Further, the isolation of mutant proteins was difficult and studies of mutants seemed out of the question because ribosomal RNAs are coded for by many genes.

The only glimmers of light in the gathering darkness were the existence of techniques for purifying ribosomal proteins and for assembling the particles from their components *in vitro*. But the

results of early efforts to analyse the relationship between ribosome structure and function using these tools were discouraging and seemed unlikely to result in the kinds of insights that attract attention, let alone new recruits. So it was that the ribosome was removed from the centre-ring position it had enjoyed in 1969, and by 1975 was to be found occupying a booth in the sideshow.

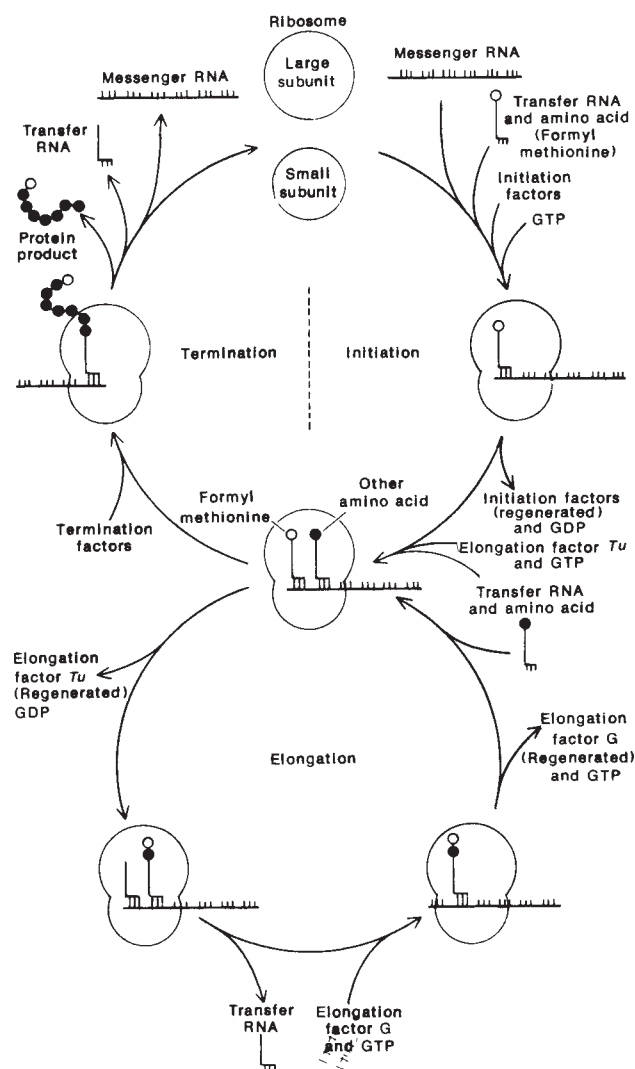


Fig. 1 The ribosome cycle. This diagram shows the ribosomal steps in protein synthesis, approximately as understood in 1969. The process starts at the top and proceeds clockwise through the steps of initiation. The lower part of the figure of eight corresponds to peptide chain elongation and is traversed repetitively in an anticlockwise direction. The left limb of the top circle represents the events of protein chain termination and release. The 1987 version of the same events would differ only in detail. [Reproduced from ref. 32 copyright 1976 by *Scientific American*.]

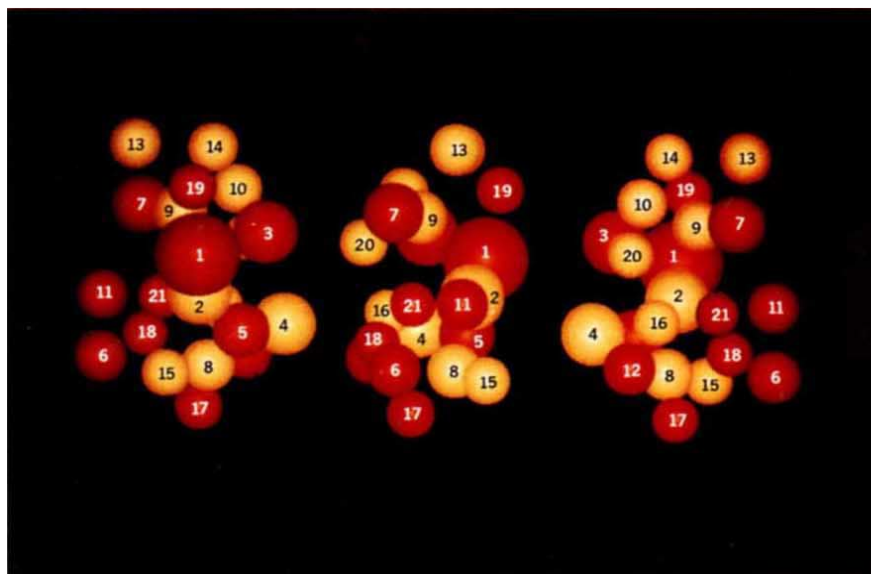


Fig. 2 The spatial arrangement of proteins in the 30S ribosomal subunit from *E. coli*. The model of the placement of proteins in the 20S subunit derived from neutron scattering measurements is shown from the cytoplasmic side (left), edge on (middle), and the subunit interface side (right)¹⁷. Proteins are depicted, for convenience, as spheres whose volumes are to scale, and whose centres are at the positions determined for protein centroids by scattering data. The numbers on the spheres designate the proteins to which each corresponds². The array is about 190 Å from the top of S13 to the bottom of S17. [From manuscript in press: M. S. Capel, M. Kjeldgaard, D. M. Engleman & P.M. Moore, *J. molec. Biol.*]

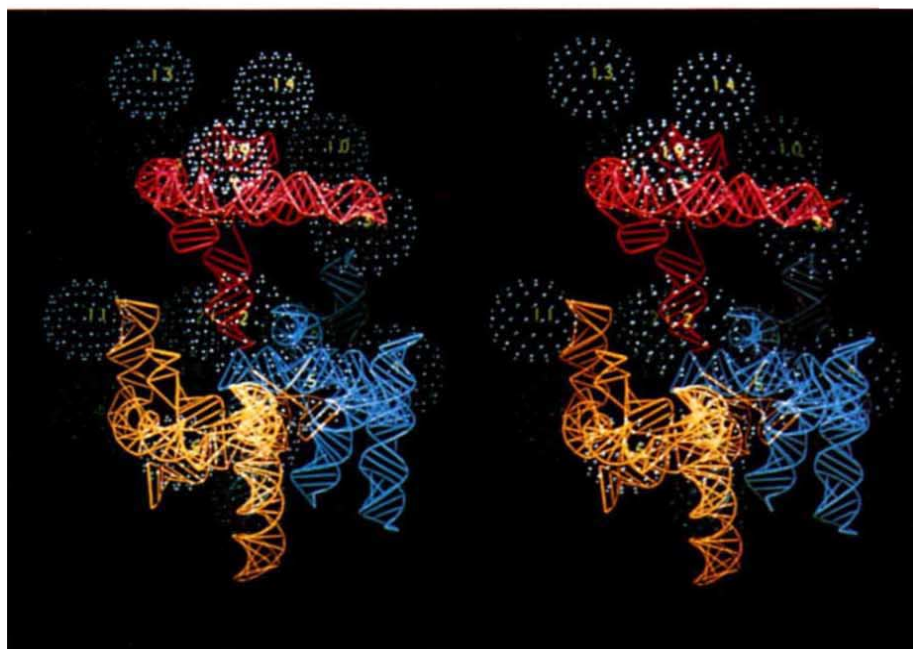


Fig. 3 16S RNA merged with the neutron map. A stereo pair is shown. The 60 per cent of the 16S RNA sequence whose placement in the 30S subunit is most firmly established, has been built into the neutron map. Components of the 3'-, central- and 5'-domains of 16S RNA (see Fig. 5) are shown in blue, yellow and red, respectively. Their spatial autonomy is clear (S. Stern, B. Weiser & H. F. Noller, unpublished data).

Nonetheless, many interesting things happened during the time the field was out of the limelight. For example, the 52 ribosomal proteins from *E. coli* were sequenced², self-assembly of the large ribosomal subunit was demonstrated³, ribosomal proof reading was recognized and explored⁴ and progress was made in elucidating ribosomal quaternary structure by a variety of innovative techniques, including immune electron microscopy (IEM)^{5,6} and neutron scattering⁷. These accomplishments, and many others equally worthy, did not command the attention they would have received in the 1960s because their implications for biochemistry as a whole seemed limited.

Renaissance

What the field has regained in the last five or six years is the aura of biological relevance it enjoyed 20 years ago. Its revitalization can be traced to two developments: the discovery of RNA enzymes and the application of cloning and sequencing technology to the study of the ribosomal RNAs.

It would be hard to overstate the impact of the discovery of RNA enzymes on ribosome biochemistry. In 1982, Cech and his coworkers announced that the initial ribosomal RNA transcript of the protozoan, *Tetrahymena*, contains an intervening sequence that it removes from itself by a series of self-catalysed, transesterification reactions⁸. In 1983, Altman and Pace and their colleagues demonstrated that the RNA portion of the ribonucleoprotein enzyme, RNase P, is the source of its nucleolytic activity⁹.

It had long been suspected that the RNA component of the ribosome plays an active role in its activity¹⁰. Otherwise, why was it there? Furthermore, there are many reasons for believing that ribosomal proteins contribute little to basic ribosomal functions¹¹. For example, many can be omitted from ribosomes, one at a time, *in vitro* or *in vivo*, without causing protein synthetic capability to collapse. The function of these proteins seems to be limited to the assembly and/or stabilization and/or optimization of the ribosome. In fact, there is no evidence that proteins

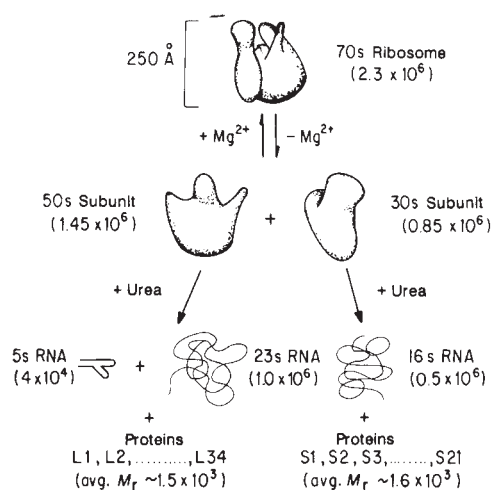


Fig. 4 The macromolecular composition of the ribosome from *E. coli*. The progressive decomposition of the ribosome into its macromolecular constituents by removal of Mg^{2+} ions and the subsequent addition of denaturants is shown. The shapes assigned the intact particle, and its subunits are those of Lake⁵. Note that many of the species obtained are named according to their approximate sedimentation coefficients. Relative molecular masses are given in parentheses (see ref. 2).

contribute essential functional groups to any of the ribosome's active sites; they may all have functions like those of the ones that can be omitted. (This assessment is not as negative as it may sound. Organisms carrying a normal complement of ribosomal proteins have a great selective advantage over those that do not.)

The arguments above notwithstanding, before 1982 there was neither firm precedent nor solid, positive evidence to suggest how RNA might contribute to the ribosome's catalytic activity, beyond providing binding specificity in interactions with other RNAs, so such suggestions were certain to be regarded with scepticism. (An example of an rRNA contribution to binding specificity was already known: the Shine-Dalgarno interaction, which ensures that prokaryotic ribosomes initiate translation at appropriate sites on messenger RNAs. This is the product of base-pairing between the sequences upstream of translational start sites and a short sequence at the extreme 3'-end of 16S RNA¹².)

The self-splicing property of pre-rRNA from *Tetrahymena* proved that RNAs can catalyse the breakage and formation of covalent bonds, and RNase P is an example of a ribonucleoprotein in which the RNA, rather than the protein, is the catalytically active entity. Thus the precedents that the ribosome field had lacked for so long were supplied and the doctrine of rRNA supremacy was transformed from a tenable hypothesis into an article of faith. Furthermore, the existence of RNase P proved that ribonucleoprotein enzymes constitute a class with a membership of at least two. Several recruits seem likely to join it soon, including the small nuclear ribonucleoprotein particles (snRNPs) that are responsible for mRNA splicing in eukaryotes. The ribosome no longer stands alone.

The discovery of RNA catalysts also led to the rapid dissemination of another theory: that early life depended upon RNA chemistry, rather than protein chemistry. RNA is clearly capable of serving as a repository of genetic information, as it performs that function in many viruses. It is now plausible that RNAs could also catalyse their own replication, something proteins seem unlikely to be able to do. Viewed in this light, the ribosome and the other RNA enzymes, however many there may be, are relicts of that earlier, RNA-based biosphere, the chemical logic of which can be understood only through the study of their mechanisms. Whatever the merits of the case, ribosome biochemistry benefits from interest in the role of RNA in evolution.

The ribosome is the ribonucleoprotein enzyme whose function is biologically most important and its mechanism is the one the advocates of the RNA hypothesis need most to understand.

Three-dimensional organization

Because of the advances in genetic engineering in the last decade, it is easier to work with rRNA today than it is to deal with ribosomal proteins, the reverse of the situation that prevailed in 1969. The first fruits of this technology were sequences for the large rRNAs, which began to appear in the late 1970s. Comparative sequence analysis soon led to secondary-structure models for both of the large rRNAs, which have been refined as the data base has grown¹³⁻¹⁵. There is no longer any doubt about the secondary structures of the rRNAs (see for example Fig. 5), or that they have been strongly conserved throughout evolution. The ribosomes in *E. coli* are indeed quite similar to those in the elephant (*Loxodonta africanus*), something that differences in their overall molecular weight and sequence might have led one to question.

Our understanding of the spatial organization of the ribosome has also benefited from improvements in nucleic acid technology. The effort to define the locations on rRNA where proteins interact, which began in the 1970s, is now being brought to a conclusion using footprinting and primer extension methods (for examples see refs 15 and 16). At the same time, studies on the spatial arrangement of proteins in the subunits have reached an advanced state. A complete model for protein placements in the small ribosomal subunit of *E. coli* has emerged from neutron-scattering studies^{17,18} (Fig. 2), which is supported by extensive data obtained by both IEM and protein-protein cross-linking studies^{5,6,19}. Similar studies of the large subunit are underway but neither the IEM data nor the neutron data are as complete as they are for the small subunit^{20,21}.

A low resolution model for the organization of the ribosomal subunits is emerging as our expanding knowledge of the positions of the protein-binding sites on rRNAs is combined with the secondary structure of the rRNAs and information about the spatial locations of the proteins (see for instance refs 22 and 23). Figure 3 shows an example of such a merger. It specifies the positions of the 16S RNA secondary-structure elements that are most firmly constrained by the available data, relative to the neutron map. About 60% of the RNA molecule is accounted for and most of the sequences known to be functionally interesting are included²³.

Ribosome structure and function

Low-resolution, three-dimensional models of the ribosome should permit the existing chemical data on the identities of components in the active sites of the ribosome to be interpreted. Neither the structural models nor the structure-function data are complete enough, however, to permit the construction of a consistent picture of the ribosomal phase of protein synthesis. On the other hand, knowledge of the spatial locations of functionally important RNA sequences has already yielded insight into the mechanism whereby streptomycin disrupts the fidelity of translation (for example see ref. 24).

Not least of the gifts of genetic engineering to ribosomology is the capability to study the effects of sequence changes on rRNA function. Two elegant examples have been provided recently by Jacob, Santer and Dahlberg²⁷, and Hui and de Boer²⁸. Both groups have altered residues at the 3'-termini of 16S RNA genes carried on plasmids, and have demonstrated that the ribosomes made from the products of these genes differ markedly from wild-type ribosomes in the efficiency with which they translate mRNAs with different Shine-Dalgarno sequences. Thus, these experiments complete the proof of the Shine-Dalgarno hypothesis and show how to do mutational experiments on rRNAs *in vivo*. Mutational experiments *in vitro* are also possible²⁹.

There is reason to hope that sequence manipulation may allow

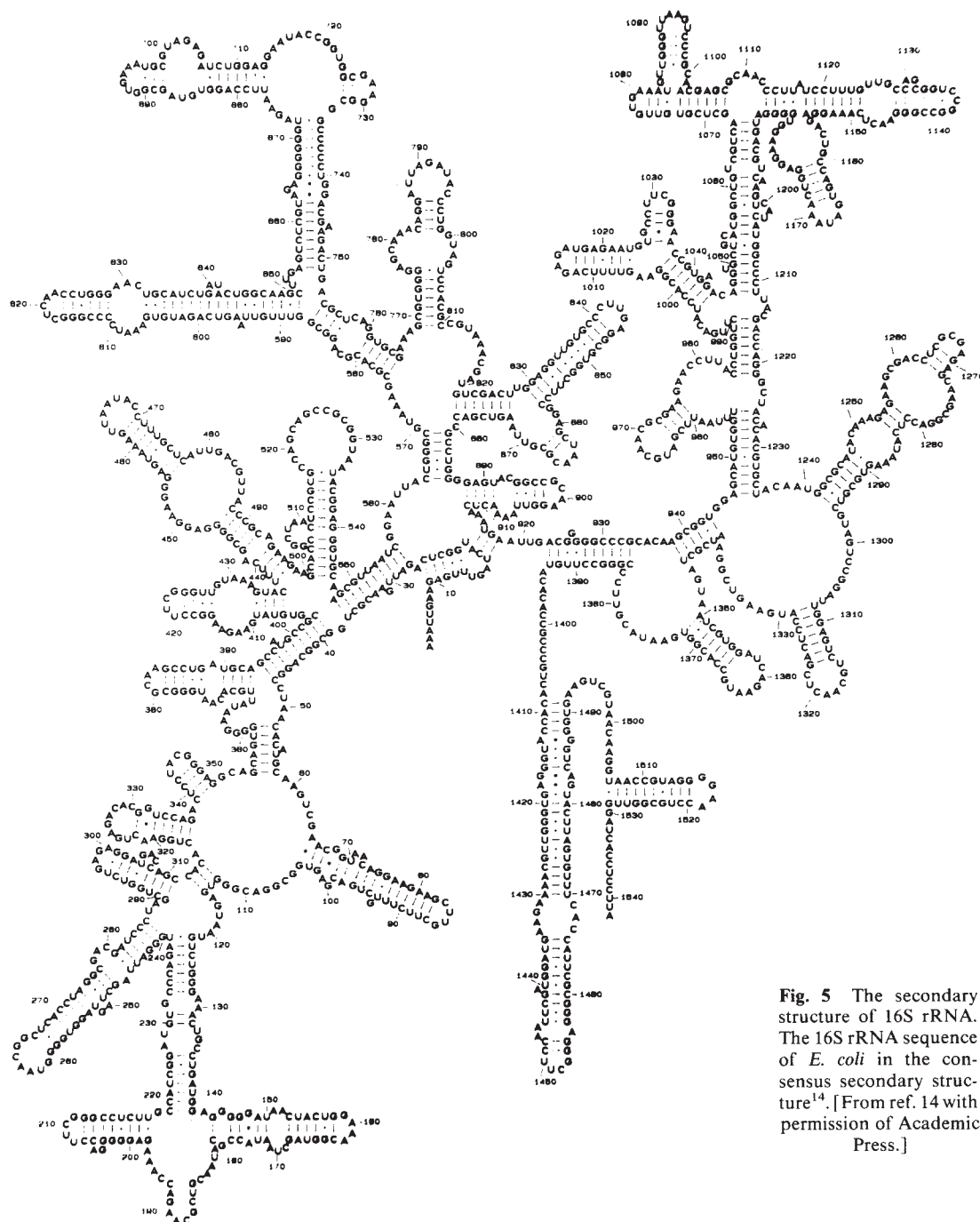


Fig. 5 The secondary structure of 16S rRNA. The 16S rRNA sequence of *E. coli* in the consensus secondary structure¹⁴. [From ref. 14 with permission of Academic Press.]

us to determine just what it is that RNA does in the ribosome. For example, there is not much difference chemically between the trans-esterification reactions catalysed by the *Tetrahymena* rRNA precursor and the transpeptidations that the ribosome catalyses during protein synthesis. Why should RNA chemistry not be the basis for the peptidyl transferase activity²⁵? The translocation step in protein synthesis, which is catalysed by the elongation factor G (see Fig. 1, bottom), is also an activity intrinsic to the ribosome²⁶. Might it not be the product of that most elusive of nucleic-acid phenomena, a 'conformational switch'? These ideas are now eminently testable.

The short-term agenda is straightforward. The relationship of rRNA sequence to ribosome function will be explored, and the three-dimensional organization of the particles worked out in as much detail as possible. It is reasonable to hope that this work will lead to a clear, qualitative understanding of the functional role of rRNA and to a low resolution, spatial understanding of the ribosomal events in protein synthesis.

High resolution structure

In the longer term, however, the need for high resolution information about ribosome structure is clear. The progress made by Yonath and collaborators in their crystallographic investigations in the past year is encouraging in this respect. They recently proposed a startling model for the 50S ribosomal subunit in which they postulate a large channel through the centre of the particle³⁰. This model was based on a three-dimensional reconstruction from electron micrographs of crystalline sheets of ribosomal subunits from *Halobacterium marismortui*, a bacterium that lives only in saturated, or near-saturated salt solutions. It would be interesting to know whether this radical model is consistent with, for example, the solution-scattering data available for large subunits from *E. coli*. More significant than this model, however, is the fact that Yonath and collaborators have discovered how to control the radiation damage which has previously limited the amount of X-ray data they

could collect from their three-dimensional crystals. This, coupled with their progress with heavy-atom labelling strategies, raises the hope that a crystallographic structure for the ribosome may soon become available³¹.

This is a stimulating time to be working on the ribosome. The answers are not all in but enough of them should materialize soon enough to maintain excitement in the field. Because of the

involvement of RNA in ribosome function and evolution, the enzymology that emerges is likely to be both novel and interesting. The ribosome has returned.

I thank Dr H. F. Noller for providing me with Fig. 3, the current version of his 16S tertiary structure map. This work was supported by the NIH.

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ARTICLES

Surface features on the nucleus of comet Halley

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Images taken by the Halley Multicolour Camera on board the ESA spacecraft Giotto during its fly-by of comet Halley in March 1986 have been improved by careful calibration and image processing methods. Surface features a few hundred metres in dimension are now visible. Several surface structures are presented in detail and briefly described.

IMAGES taken by the Halley Multicolour Camera (HMC) on board the European Space Agency (ESA) spacecraft Giotto during the encounter on 13 March 1986 show clear details of the nucleus of comet Halley. Even the dark side was silhouetted against a background of illuminated dust. The first results, summarized immediately after the encounter¹, were that comet Halley had one large nucleus, with visible dimensions of 15×8 km, its surface was very dark (low geometric albedo of ≈ 0.04), its sublimation activity was locally concentrated and roughly directed towards the Sun and, therefore, the ice had to be covered by a crust preventing sublimation on large parts of the surface. The temperature of the crust had to be appreciably above that of the sublimation equilibrium.

Data and processing

HMC was a telescope with 1 m focal length operating fully automatically from the spinning Giotto spacecraft (see refs 1 and 2 for a full description of the instrument). During the last 5 min before closest approach only small images of 74×74 pixels, with a corresponding field of view of $0.1 \times 0.1^\circ$, were obtained (see images 3457, 3475, 3491 and 3502 in Fig. 1). The automatic tracking algorithm of HMC concentrated on the brightest part of the object, the active region on the northern tip. More than 2,000 images were returned, the last useful one (image 3504) having been taken from 1,675 km at a resolution

of < 50 m per pixel. The extremely low signal (the observed peak signal corresponds to a reflectivity of only 0.007) and the higher-than-expected temperature of the spacecraft and instrument combined with the short exposure times (0.25-5 ms) require accurate dark current subtraction and noise removal to reconstruct the received signal³. The dynamic range of the images then approaches 1,000 (see Table 1 for a summary of images discussed in this paper).

Parallel to the radiometric calibration effort, star observations have been used to determine the point spread function⁴. HMC operated in line-scan mode. Owing to the spin of the spacecraft the field of view covered a segment of a circle, which was then stored in the rectangularly shaped detector. The images must therefore be geometrically corrected to restore the true geometry (see image 3056 in Fig. 1). The derived geometric corrections, to sub-pixel accuracy, provide a firm basis for accurate co-registration of the images. Images can be combined so that images with higher resolution but smaller geometric field of view replace the corresponding parts of earlier images. Features with high resolution are seen within the context of the whole scene (see Fig. 2). Care must be taken in interpreting these composite images, owing to varying resolution. The intensities presented are stretched, occasionally in a nonlinear manner, to expand the contrast. Another method of increasing the visibility of low-contrast features is to subtract a strongly smoothed image

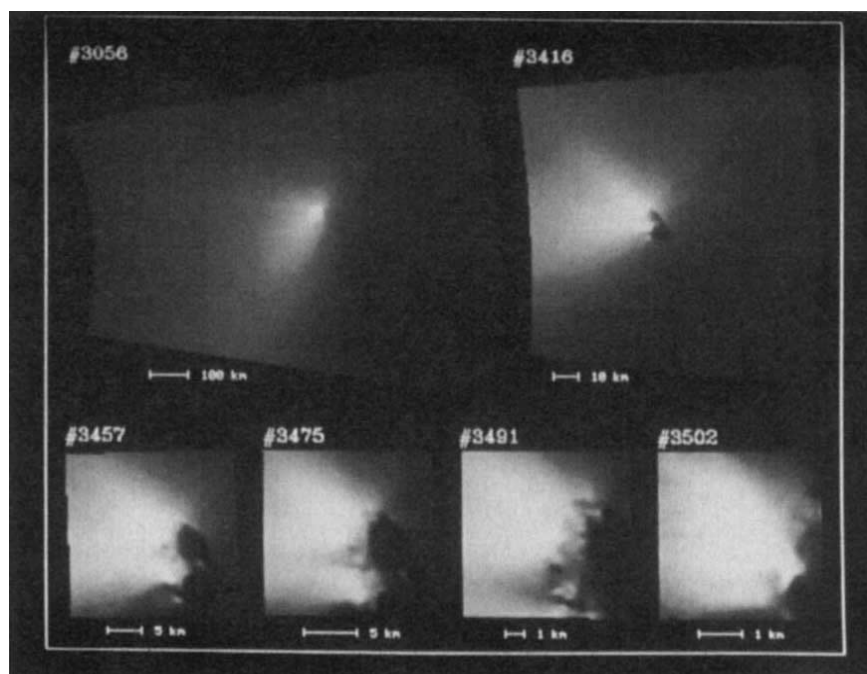


Fig. 1 Six examples of HMC images (original frame sizes) that are filtered, calibrated and deconvolved by the point spread function (PSF) (except image 3502). The Sun is on the left side, 7° below the horizontal in image 3502.

from the original, thereby reducing the large-scale brightness variations ('unsharp masking'). Figure 3 displays the various steps of processing. Earlier images of the inner dust coma can be treated similarly. The clarity of the jet features (Fig. 6) is quite spectacular, an improvement on the azimuthal shifting technique (compare the same HMC image in ref. 3).

The nucleus

Figure 1 shows examples of restored images taken during the approach to the nucleus of comet Halley (compare with images in ref. 5, in which Figs 1, 2, 4, 5 and 6 correspond to image numbers 3056, 3416, 3443, 3492 and 3502, respectively). The nucleus is clearly visible in image 3056, taken at a distance of 124,000 km about half an hour before closest approach. Note the strongly asymmetric and concentrated dust emission towards the Sun. The emission, however, is not symmetric relative to the comet-Sun direction. Images taken by the Vega spacecraft under almost equal geometrical conditions⁶ several days earlier show a strikingly similar dust distribution³.

The nucleus is peanut-shaped, and can be approximated by an ellipsoid of $16 \times 8 \times 8$ km (refs 7-9). Its long axis is tilted 30° out of the image plane (the northern tip towards the observer) causing a slight foreshortening. The rotation axis is approxi-

mately perpendicular to the long axis, pointing $\sim 13^\circ$ into the image plane. The nucleus rotates with a period of ~ 54 h around this axis (the northern tip away from the observer). For images taken minutes before closest approach the projected direction to the Sun is $25\text{--}30^\circ$ above the horizontal on the left; the Sun-nucleus-camera angle decreases from $\sim 107^\circ$ at large distances. Celestial north is approximately upward. The outline of the projected surface (Fig. 2) is clearly visible on the dark side; however, the signal to noise is rather poor, particularly on the southern end. The last image showing the complete nucleus (3457) has a resolution of 320 m per pixel.

The definition of the dark limb has been improved by addition of images. The estimated error of the outline varies from about 300 to 150 m from the southern to the northern end. The outline is well defined by the maximum gradient of the transition. The roughness of the outline indicates features of a few hundred metres in dimension.

The sunward illuminated limb is less well defined. Here the outline can be derived from the maximum of the brightness of scans perpendicular to the limb. This maximum intensity has to come from the brightening of the limb or from the dust just in front of it. The situation is somewhat more complicated in the northern active area because of the variety of jets. Only very

Table 1 HMC image summary

Image number	Frame size (pixel)	Time to encounter (s)	Distance to nucleus (km)	Scale (m per pixel)	Exp* time (μ s)	Sun azimuth (deg)	Sun elevation (deg)
3056	390×292	1,814	124,020	2,780	752	+173	17
3416	196×196	375	25,630	570	633	-154	16
3457	74×74	211	14,430	320	355	-152	15
3460	74×74	199	13,610	310	334	-151	15
3475	74×74	139	9,520	210	232	-151	14
3480	74×74	119	8,150	180	199	-150	14
3491	74×74	75	5,160	120	126	-150	11
3496	74×74	55	3,800	90	93	-150	9
3500	74×74	39	2,730	60	66	-151	6
3502	74×74	31	2,200	50	54	-151	4

* Per line; multiply by 6 for effective exposure time.



Fig. 2 A composite of six images ranging in resolution from 320 to 60 m per pixel. Some of the single images are displayed in Fig. 1.

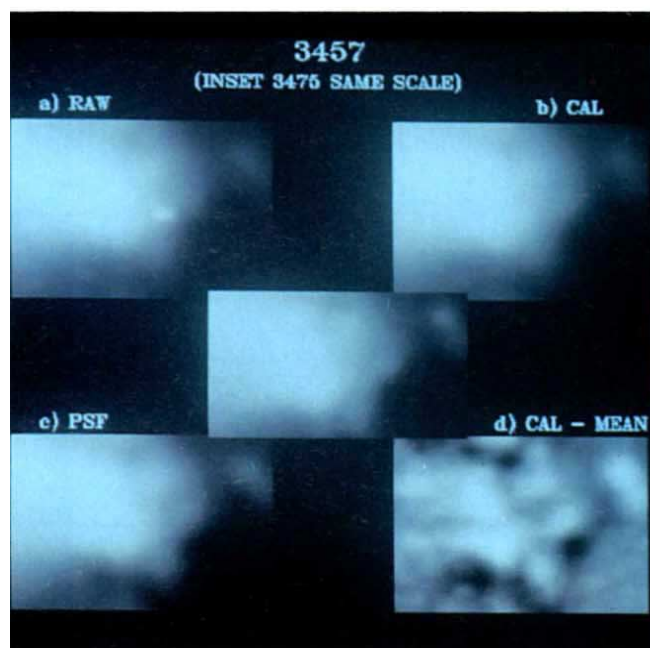


Fig. 3 The region of the central depression is used to exemplify the effects of the various steps of image processing. Upper left, raw data; upper right, dark current subtracted and response calibrated; lower left, PSF deconvolved; lower right, a heavily smoothed image (7×7 pixels) subtracted from the calibrated image (upper right) and stretched in intensity. Inset, a cut-out of image 3475 (calibrated); its resolution is better by a factor 1.5 than that of image 3457.

minor changes in slope indicate the limb at the two regions without activity next to the 'crater' and north of the small 'southern jet' (see below). The dust column above the surface changes quite strongly, decreasing away from the region of highest activity.

Surface features

Figure 2 is a composite of six images. The resolution improves from ~ 320 to 60 m per pixel. The composite can be compared to image 3457 of Fig. 1, its outermost image. In addition to the change in resolution, the aspect angle changes significantly (by 11°) during the last images. Many surface details are visible.

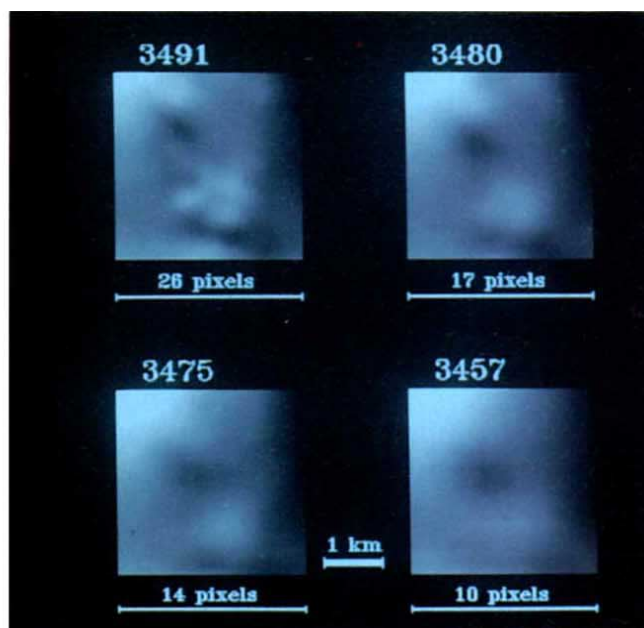


Fig. 5 The effect of resolution demonstrated on the crater.

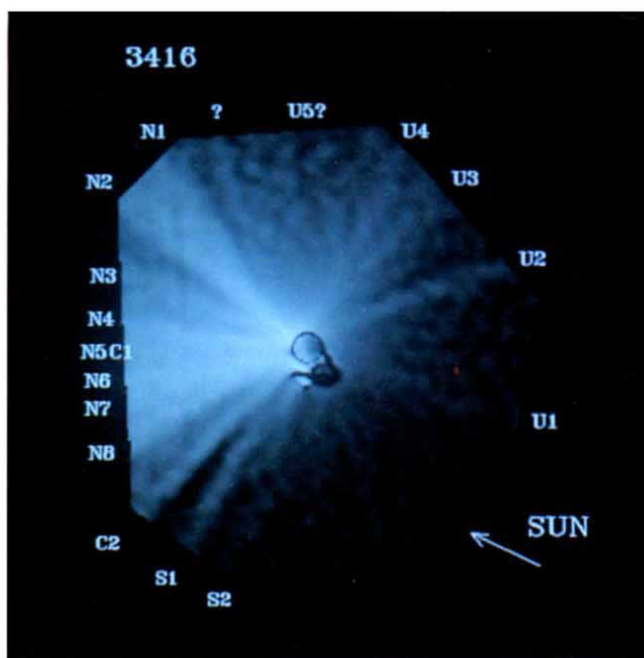


Fig. 6 Image 3416 processed to show the various dust jets and filaments. Nomenclature according to Thomas and Keller¹¹.

The most prominent details are displayed in enlargements in Figs 3 and 4. The perimeters of the cut-outs are indicated in Fig. 4a. The intensities of the cut-outs are individually stretched, increasing their contrast. The bandwidth of the clear filter is 374 nm. In addition, intensity surface plots are displayed. The projected view directions for these plots are indicated, as well as the directions towards the Sun and celestial north. 25% of the projected nuclear surface is insolated. No features can be detected on the dark side.

The 'finger' (Fig. 4b). The northern tip of the nucleus is not yet illuminated by the Sun. However the morning terminator is interrupted by a bright feature (an outcropping or hill, perhaps)

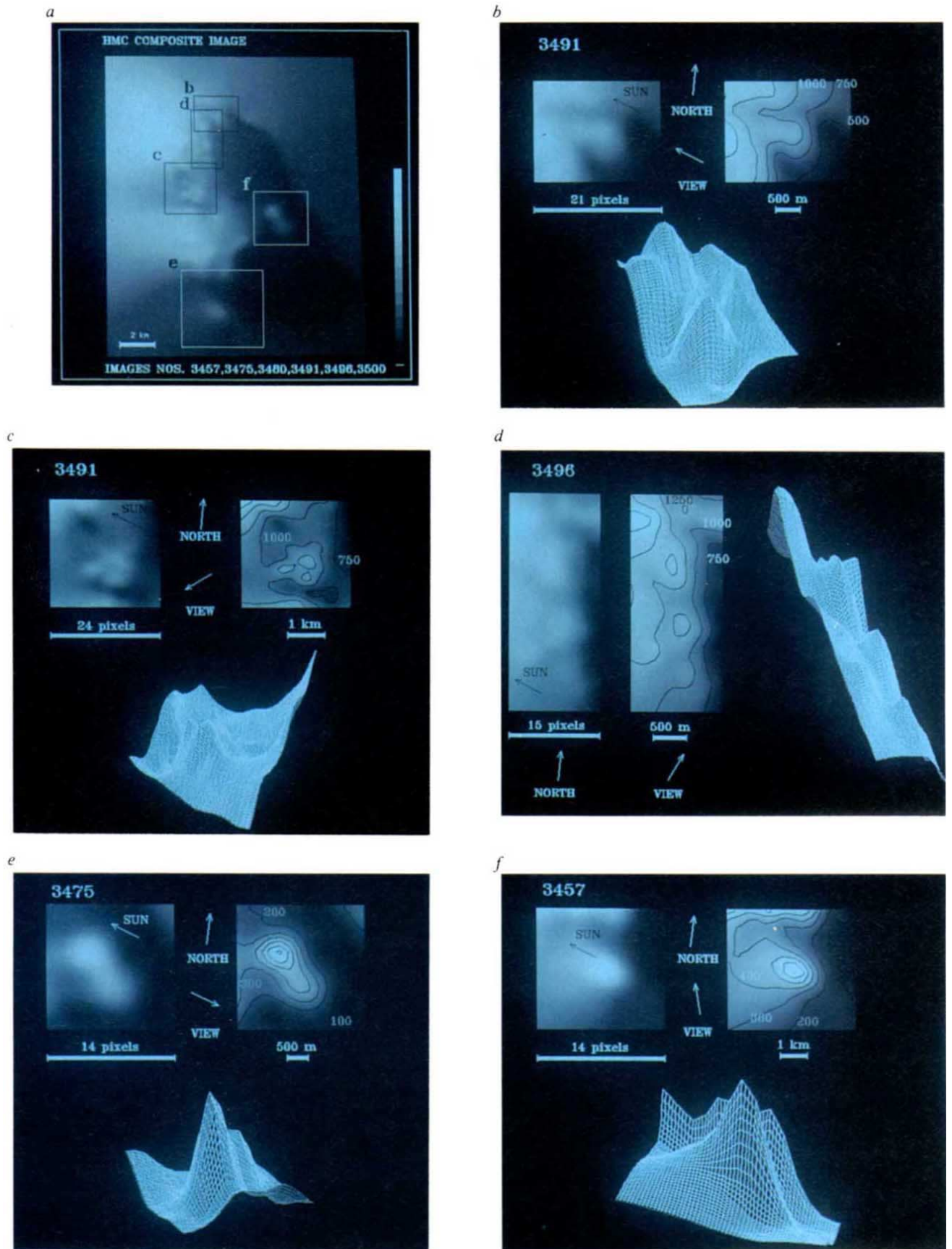


Fig. 4 *a*, Frames of the cut-outs for: *b*, finger; *c*, crater; *d*, chain of hills; *e*, southern jet; *f*, mountain. Isophotes in *b*-*f* are identified in $\text{mW m}^{-2} \text{sr}^{-1}$.

separating a small 'finger-like' patch from the main area of the dark surface. This is a good example of the large-scale roughness of the surface. The width of the bright feature is ~ 600 m; the length of the 'finger' is 1.4 km, its width being ~ 550 m next to the bright feature.

The 'crater' (Fig. 4c). This roundish feature is one of the most prominent structures and is visible in the raw data. Preliminary photoclinometric analysis¹⁰ shows that the 'crater' is $\sim 2,000$ m in width and rather shallow, being ~ 150 m in depth. The dust column above this area contributes an unknown amount of light to the observed signal. The shape is appreciably distorted, as its sunward rim seems to be close to the limb of the nucleus. Figure 5 shows the effect of resolution. Morphologic substructures are clearly visible in images taken from closer than 8,000 km (image 3480). The rim and the bottom of the 'crater-like' feature are structured. It is not clear whether the 'crater' is really a coherent feature. This area may have been associated with particularly strong activity, if not in this return then in previous apparitions.

The 'chain of hills' (Fig. 4d). The terminator running almost north-south on the northern part of the nucleus is marked by a series of bright-dark contrasts. Each bright patch has its dark counterpart. At least four 'hills' are well resolved, with dimensions of ~ 500 m. A size of ~ 0.5 km is found for many features, in some cases even in regions where the resolution is quite sufficient to yield smaller features (compare with image 3502, Fig. 1). The hills are rather regularly spaced; the peak intensities are separated by distances of 800 m, 1 km and 800 m. The second hill in the chain (from the north) is 1.2 km from the bright feature forming the 'finger'.

The 'southern jet' (Fig. 4e). The terminator reaches the sunward limb of the nucleus at its southern end. A small area of activity is isolated. The isophotes centred on the bright spot are pointing towards the Sun, although two relatively faint dust filaments¹¹ are directed southward (see also features S1 and S2 of Fig. 6). The rather strong gradient towards the dark side of the nucleus north of the active area could indicate that this part of the nucleus is in the foreground, masking parts of the sunlit region. Here, the course of the limb is uncertain.

The 'mountain' (Fig. 4f). Probably the most prominent feature is the bright, rather sharply defined patch approximately in the centre of the visible nuclear surface. The terminator recedes in this central part, indicating a larger effective curvature of the nuclear body caused by the 'central depression' (see below). At its end, however, separated by a small dark strip, the brightness suddenly increases with the sharpest gradient found anywhere in the images. The spot is apparently unresolved, allowing the determination of an upper limit on its dimension (≤ 0.2 km). If this is the top of a hill illuminated by the morning sunlight then its height could be roughly estimated to be 0.5–1 km (ref. 12).

The 'central depression' (Fig. 3). This area is located right in the middle of the elongated nucleus and laces it almost like a waist. A broad valley (causing a larger effective radius of the nucleus at this area) tapers from the limb towards the mountain. The region of the limb lies close to the north pole of the nucleus and the Sun is circumpolar at this high nuclear latitude¹³. The surface looks smoother than the illuminated northern region. This is partly due to the inferior resolution. However, the images with the highest available resolution displayed for highest

contrast show structures of similar coarse roughness as the northern parts.

Activity

Dust emission is concentrated at certain parts of the nucleus. By far the strongest visible active area is located on the northern sunward tip of the nucleus. The activity level at the central depression (Fig. 3) is considerably lower. High-resolution images show fine structures in the global dust streamers (see image 3502 of Fig. 1). They can in some cases be followed out to more than 100 km. These dust filaments¹⁴ are minor enhancements ($\sim 30\%$) in the dust density and are ~ 0.5 km across near the nucleus. They are strongly collimated, with a cone angle of $< 10^\circ$. The source regions of some filaments can be identified, for example, those of filaments N3 and N4 (for identification of the filaments see Fig. 6). Other bright spots close by do not show signs of particularly high activity. Seventeen dust filaments can be seen in Fig. 6. The strongest dust jets (N1, N2, N6 and N8) seem to come from parts of the surface of the northern active region not visible for the Halley Multicolour Camera, as might be expected considering the illumination angle and the direction of rotation. Some of the filaments cross each other in front of the northern active area. The relatively weak filaments pointing away from the Sun in the projection on the image (labelled U) could originate from the still-insolated parts of the surface near the evening terminator.

Summary and outlook

The strongly non-spherical shape of the nucleus cannot have been produced by sublimation (6 m per orbital revolution³). If the present nucleus is not a remnant of a break-up of a much larger nucleus, its shape had to be formed during its creation process. An irregular shape most probably results from the agglomeration of rather large sub-nuclei. The surface details and the variation of the limb give the impression of a hilly surface with a coarse roughness at least down to a scale of 0.5 km. Smaller scales could only be visible around the active northern area where the contrast is low. This scale size of 0.5 km could be inherent and is possibly characteristic of 'building blocks'.

The images taken by the Halley Multicolour Camera during the Giotto fly-by of comet Halley have not only revealed the existence of one large cometary nucleus but have also shown a wealth of detail. Further improvements and refinements could be expected from the separation of the scattered light of the dust above the nuclear surface from the reflected light. This task may be successfully attacked using the corresponding images taken through the broad-band colour filters. The interpretation of the nucleus features hinges on the comprehension of the three-dimensional orientation of the nucleus and of its surface and activity. The continuing collaborative effort between the imaging teams of the Vega and Giotto spacecraft aims at establishing a three-dimensional model of the nucleus.

A large team of scientist and technicians have been involved in the success of the Halley Multicolour Camera. The quality of the presented images is based on the calibration (contributions of A. Delamere and H. Reitsema are particularly acknowledged) and on the image processing software (developed by E. Mikusch and G. Schwarz). We are indebted to all our colleagues on the HMC team.

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Identification of a receptor for protein import into chloroplasts and its localization to envelope contact zones

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An anti-idiotypic antibody approach was used to identify an integral membrane protein of the chloroplast envelope as a receptor for protein import into the chloroplast stroma. The import receptor is found in contact sites between the outer and inner membrane of the chloroplast envelope.

A LARGE number of chloroplast (or mitochondrial) proteins coded for by nuclear genes are synthesized in the cytoplasm and are then imported into these organelles (for review on chloroplast import see ref. 1). The small subunit (S) of ribulose-1,5-bisphosphate carboxylase (RuBPCase) was the first of these organellar proteins whose synthesis and import was studied in detail. It was first observed for S of *Chlamydomonas reinhardtii* that it is synthesized as a larger precursor, pS (ref. 2), containing an NH₂ terminal extension, termed transit peptide³. It was also shown that pS is synthesized on free rather than envelope-bound ribosomes, suggesting a facultatively post-translational mode of protein uptake² as opposed to the previously observed and presumed obligatory cotranslational (translation-coupled) mode of translocation of secretory proteins across microsomal membranes by membrane-bound ribosomes⁴. In an *in vitro* import system it was shown that *in vitro* synthesized pS from pea could indeed be imported post-translationally into isolated pea chloroplasts^{5,6}. During or shortly after import, the transit peptide is removed by a specific protease in the stroma^{2,3,7-9}.

One of the major unresolved problems of protein import into chloroplasts (or mitochondria) is the mechanism of uptake. Is there, as was suggested as early as 1977, a receptor, "possibly located in regions where the inner and outer envelope membranes are in contact" (ref. 2), which recognizes the transit sequence? Or does the transit sequence somehow probe the unique lipid composition of the chloroplast envelope and traverse the chloroplast envelope membrane unaided by proteins as suggested, for example, in ref. 10 in one of the models for protein import into mitochondria? Evidence in favour of a proteinaceous receptor or receptors for protein import into chloroplasts is so far based solely on the finding that *in vitro* import is abolished when chloroplasts are subjected to protease treatment before import^{11,12}. Here we have identified an integral membrane protein of the chloroplast envelope as a receptor for protein import and have localized this receptor to contact zones between the outer and inner membrane of the chloroplast envelope.

Anti-idiotypic antibodies inhibit import

The idiotypic^{13,14} network theory of Jerne¹⁵ proposes that 'idiotypes' (antigenic determinants associated with the region in or near the antigen-binding site of an antibody) can act as immunogens; antibodies that recognize them are referred to as anti-idiotypic antibodies. Anti-idiotypic antibodies have been used in the past to identify receptors (for review see ref. 16). The underlying principle of this approach is as follows. Antibodies are generated against the ligand; among these are antibodies that recognize the ligand in a way that mimics the physiological receptor-ligand interaction. Consequently, the idiotypes of some of the antibodies against the ligand may have structures in common with the receptor. A second set of antibodies raised against the 'receptor-like' idiotypic determinants

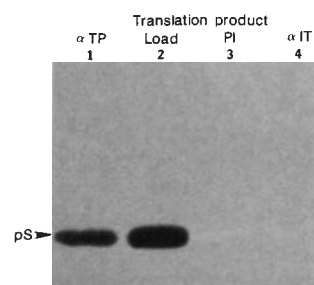


Fig. 1 Immunoprecipitability of pS by various antisera. Newly synthesized pea pS (precursor of small subunit (S) of RuBPCase) (lane 2) was subjected to immunoprecipitation using either transit peptide antiserum (α -TP) (lane 1), or preimmune serum (PI) (lane 3) or anti-idiotypic antiserum (α -IT) (lane 4).

Methods. Translation of plasmid-transcribed mRNA in a cell-free wheat germ system²⁷, immunoprecipitation²⁹ and analysis by SDS-PAGE and autoradiography²⁷ was as previously described. A 30 amino-acids-long peptide representing the carboxyl-terminal portion of the transit peptide of pea pS up to the ultimate Cys¹⁷ was chemically synthesized, and antibodies were produced in rabbits as previously described³⁰. An IgG fraction³¹ of the anti-transit peptide antiserum was used to prepare rabbit anti-idiotypic antiserum following a published procedure³². Preimmune sera were obtained from rabbits before injection of the transit peptide.

of the anti-ligand antibodies contain anti-idiotypic antibodies that recognize the ligand-binding site of the receptor.

We raised antibodies against a chemically synthesized peptide representing a 30 residue-long carboxyl-terminal portion of the transit peptide of pea pS (ref. 17). These antibodies immunoprecipitate pea pS synthesized in a wheat germ cell-free translation system programmed with plasmid-transcribed messenger RNA (Fig. 1, lanes 1 and 2), but not mature S (data not shown). Preimmune serum does not react with pS (lane 3). A second set of antibodies raised against the transit sequence antibodies and presumably containing anti-idiotypic antibodies directed against the transit sequence-binding site of the putative import receptor(s) did not, as expected, immunoprecipitate pS of pea (Fig. 1, lane 4).

Interestingly, addition of decomplexed serum containing the presumptive anti-idiotypic antibodies to a suspension of isolated chloroplasts resulted in visible aggregation and precipitation of the chloroplasts whereas addition of the corresponding decomplexed preimmune serum had no effect (data not shown). These observations suggested that the aggregation/precipitation of chloroplasts was specific and that this second antiserum contained antibodies directed against a domain located on the surface of the chloroplast, presumably the ligand-binding site of the import receptor(s).

This precipitation precluded use of the IgG derived from the

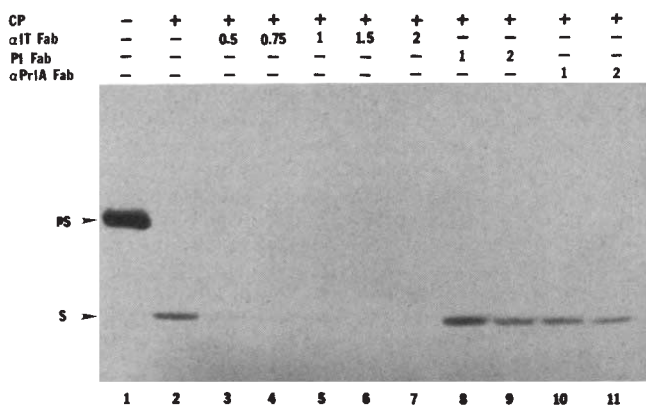


Fig. 2 Effect of Fab fragments of various IgGs on *in vitro* import of pea pS into pea chloroplasts. A wheat germ translation mixture (0.25 μ l) containing newly-synthesized pS of pea (lane 1) was incubated in a total volume of 0.3 ml for 15 min at room temperature with isolated pea chloroplasts (CP) equivalent to 20 μ g chlorophyll that were preincubated in the absence (lane 2) or the presence of various amounts (150–600 μ g) of Fab fragments (lanes 3–11). Numbers on top indicate the final concentration of Fab in mg ml^{-1} .

Methods. Chloroplasts from pea leaves were isolated as previously described²⁷. IgG fractions³¹ from various decomplexed (56 °C for 20 min) rabbit sera (α -IT, anti-idiotypic; PI, preimmune obtained from rabbits before injection of anti-transit peptide IgG; α PrlA, antiserum against a synthetic peptide corresponding to the 20 N-terminal residues of *Escherichia coli* PrlA protein (Watanabe and Blobel, unpublished) were isolated and used to prepare monovalent Fab fragments as described³³. The basic import reaction was essentially as previously described²⁷ with the following modifications. Chloroplasts were preincubated in the absence or presence of various Fabs in a total volume of 0.25 ml at 4 °C for 30 min. A master mixture (50 μ l) containing pS and all other components of the import assay was added to final concentrations of: 50 mM HEPES/KOH, pH 7.7; 0.33 M sorbitol; 2 mM Mg diacetate ((OAc)₂); 40 mM KOAc; 50 μ M dithiothreitol (DTT); 5 mM methionine, 100 μ g ml^{-1} bovine serum albumin (BSA), 1 mM ATP and 0.25 μ l postribosomal supernatant containing pS. The import reaction was carried out for 15 min at room temperature and the mixture analysed as previously described²⁷.

antiserum in an *in vitro* import reaction. But monovalent Fab fragments prepared from the purified IgG did not precipitate chloroplasts (data not shown) as expected, and could therefore be tested in an import reaction, consisting of a wheat germ cell-free translation system containing newly synthesized pea pS (Fig. 2, lane 1) and post-translationally incubated with isolated pea chloroplasts. Incubation resulted in conversion of some pS (relative molecular mass 20,000; M_r 20K) to S (M_r 14K; Fig. 2, lane 2) due to import into the chloroplast stroma and removal of the transit sequence by a specific stromal endoprotease.

When increasing amounts of monovalent Fab fragments were added to the import reaction there was increasing inhibition of import (Fig. 2, lanes 3–7). At the highest concentration of Fab fragments there was >95% inhibition of import (Fig. 2, compare lane 2 with lane 7). Equivalently high concentrations of Fab fragments of preimmune IgG (Fig. 2, lanes 8 and 9) or of IgG of a non-related immune serum (Fig. 2, lanes 10 and 11) had no significant effect on import. Taken together, the data indicate that the second antiserum contained anti-idiotypic antibodies recognizing the transit sequence-binding site of the import receptor and that Fab fragments of these antibodies inhibited translocation, presumably by competing with the binding of the transit sequence of pS to its receptor.

Localization of import receptors

Immunofluorescence was performed using immobilized and slightly fixed chloroplasts and either the anti-idiotypic antiserum

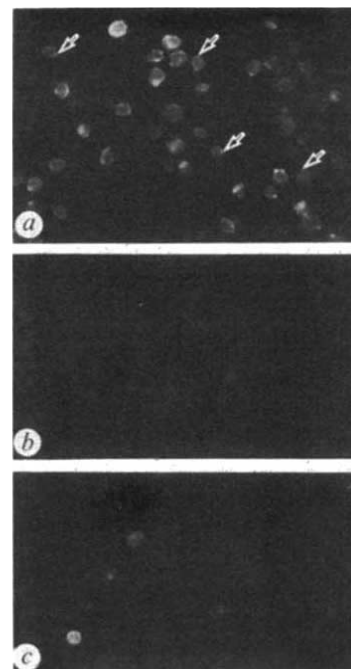


Fig. 3 Immunofluorescence microscopy of isolated and immobilized pea chloroplasts. *a*, Chloroplasts were successively incubated with anti-idiotypic antiserum and fluorescein-conjugated goat anti-rabbit IgG; *b*, preimmune serum was used instead of anti-idiotypic antiserum during the first incubation; *c*, same as *a* except that chloroplasts were treated with trypsin (40 μ g ml^{-1}) before incubation with antisera. Arrows in *a* indicate immunofluorescent patches in the periphery of the chloroplast. Magnification, $\times 1,500$.

Methods. Isolated chloroplasts (equivalent to 1 μ g chlorophyll) were allowed to settle onto a poly-L-lysine-coated polystyrene dish for 1 h at 4 °C, and immersed in an ice-cold aldehyde fixative (0.05% glutaraldehyde+0.25% paraformaldehyde) in buffer A (50 mM HEPES/KOH, pH 7.7, 0.33 M sorbitol, 40 mM KOAc, 2 mM Mg(OAc)₂) for 30 min at 4 °C. The fixed chloroplasts were washed with buffer A containing 10 mM NH_4Cl , and incubated with buffer A-BSA (2%) solution with mild agitation for 1 h at 4 °C. The samples were washed several times with buffer B (buffer A, 1% BSA, 1 mM phenyl methyl sulphonyl fluoride (PMSF)) and incubated either with decomplexed anti-idiotypic antisera (1:500) or decomplexed preimmune serum (1:500) in buffer B for 2 h at room temperature. Subsequently, the samples were washed with buffer B and incubated with goat IgG (100 μ g ml^{-1}) in the same buffer for 20 min. They were rewashed with buffer B and incubated with fluorescein-conjugated goat anti-rabbit IgG (1:200, Cappel) for 1 h, protected from light. After several washings with buffer A+0.1% BSA, a drop of *p*-phenylenediamine (1 mg ml^{-1} in 90% glycerol, pH 8.0) was placed on the sample. Coverslips were mounted, edges sealed, and the sample was examined with a Zeiss photomicroscope III. For trypsin treatment (*c*) immobilized and fixed chloroplasts (see above) were incubated with trypsin (5–40 μ g ml^{-1}) in buffer A for 15 min on ice. The samples were then washed with buffer A, 1% BSA, 2 mg ml^{-1} soybean trypsin inhibitor, 1 mM *p*-aminobenzamidine, 5 mM ϵ -aminocaproic acid and 1 mM PMSF and incubated with anti-idiotypic antiserum as described above.

(Fig. 3*a*) or a preimmune serum (Fig. 3*b*). Distinct patches of bright immunofluorescence (indicated by arrows in Fig. 3*a*) were visible in the periphery of the chloroplasts, suggesting that the import receptors are not evenly distributed over the surface of the chloroplast.

When chloroplasts were treated with increasing concentrations of trypsin before preparation for immunofluorescence the bright immunofluorescent patches at the periphery of the chloroplast gradually disappeared (data not shown). At the highest trypsin concentration (40 μ g ml^{-1}) the immunofluorescent patches were completely abolished (Fig. 3*c*) together with the ability

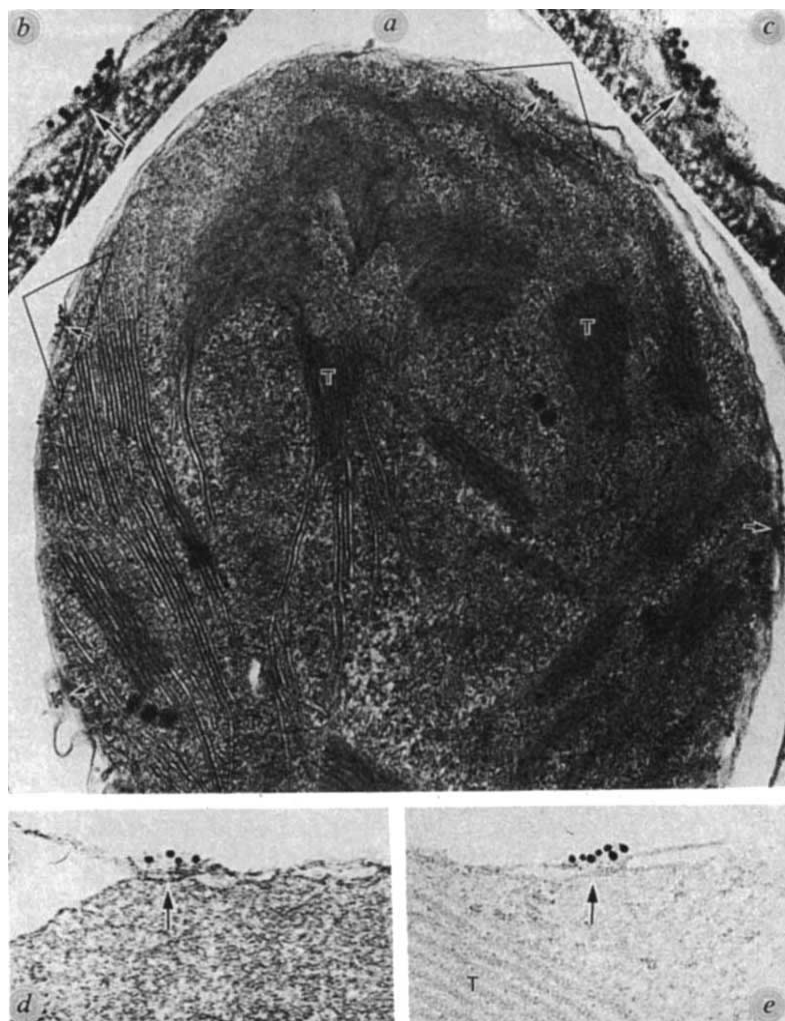


Fig. 4 Immunogold electron microscopy of isolated pea chloroplasts. Chloroplasts were immobilized, fixed and successively incubated with anti-idiotypic antiserum and goat anti-rabbit IgG conjugated to colloidal gold. *a*, Immunogold particles are seen at the contact sites (arrows) between inner and outer chloroplast envelope membranes; *b*, *c*, higher magnification micrographs of the regions enclosed by the triangular insets in *a*; *d*, *e*, higher magnification electron micrographs taken from other chloroplasts. T stacks of thylakoid. Magnifications: *a*, $\times 56,000$; *b*–*e*, $\times 150,000$.

Methods. Immobilized chloroplasts (see Fig. 3 legend) were incubated with 50 mM HEPES/KOH, pH 7.7, 0.66 M sorbitol for 1 min at 4 °C, then with an ice-cold aldehyde fixative (0.05% glutaraldehyde + 0.25% paraformaldehyde) in the same buffer for 30 min at 4 °C. After washing with buffer A (see legend to Fig. 3) containing 10 mM NH_4Cl and buffer A plus 2% BSA, the samples were incubated with decomplexed anti-idiotypic rabbit antiserum (1:500) in buffer B (see legend of Fig. 3) for 2 h at room temperature, washed and incubated with goat IgG (100 $\mu\text{g ml}^{-1}$) in buffer B. The samples were washed as above and incubated with goat anti-rabbit IgG conjugated to colloidal gold (10 nm, 1:50, Janssen). The samples were washed with buffer A, immersed in Karnovsky's aldehyde fixative containing 0.33 M sorbitol (1 h), osmicated (1 h), stained with uranyl acetate (2 h) and dehydrated in graded series of ethanol as described³⁴. The samples on the plastic dishes were scored with a scalpel, flushed off with propylene oxide, and pelleted by centrifugation. The pellets were immersed in an EPON:propylene oxide mixture (1:1), infiltrated with pure EPON and embedded. Ultra-thin sections (60 nm) were mounted on copper grids and stained with uranyl acetate and lead citrate³⁴. The samples were examined with a JOEL 100 CX electron microscope at an accelerating voltage of 80 kV.

of the chloroplast to import (data not shown), indicating that the ligand-binding site of the import receptor(s) is sufficiently exposed at the surface of the chloroplasts to be accessible to degradation by trypsin, in agreement with earlier reports^{11,12}.

To localize the import receptor more precisely we performed immunogold electron microscopy using preimmune serum or the anti-idiotypic antiserum (Fig. 4). With the preimmune serum (data not shown) there was no immunogold decoration of the chloroplast. With the anti-idiotypic antiserum, there was decoration of patches on the chloroplast surface. At higher magnifications, these patches can be seen to coincide with the contact zones¹⁸ where outer and inner envelope membranes are closely apposed to each other (Fig. 4*b–e*). Interestingly, Neupert and coworkers^{19,20} have recently identified translocation intermediates in protein import into mitochondria¹⁹ and have provided evidence that these intermediates are located in contact zones²⁰.

Identification of the import receptor

To determine which of the polypeptides of the chloroplast react specifically with the anti-idiotypic antibodies, polypeptides of either whole chloroplasts or of chloroplast subfractions, representing the soluble stroma and membrane fraction, were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose and probed either with preimmune serum or anti-idiotypic antiserum (Fig. 5). None of the chloroplast polypeptides reacted with the preimmune serum (lane 2), but two chloroplast proteins of M_r 52K and 30K reacted with the anti-idiotypic antibodies (lane 3).

The bulk of the immunoreactive 52K polypeptide was found in the stroma subfraction (lane 4) and coincided with the major

Coomassie blue-stained polypeptide (not shown) of this fraction, suggesting that the immunoreactive 52K polypeptide might be the large subunit of RuBPCase. In fact, when 18S RuBPCase containing only large and small subunits of the enzyme was purified²¹ from pea chloroplast lysate by ion-exchange chromatography followed by sucrose gradient centrifugation and tested by Western blot, the anti-idiotypic antiserum did react with the large subunit (lane 8). One possible and interesting explanation of this is that within the 30 C-terminal residues of the synthesized transit peptide there is some critical information specifying interaction not only with the import receptor but also with the large subunit of RuBPCase. Thus, the anti-idiotypic antiserum would contain antibodies to both of these components. The large subunit of RuBPCase is synthesized within the chloroplast, and is known to interact with a binding protein which presumably maintains it in a soluble form until it associates with the small subunit to form the 18S holoenzyme complex²². Furthermore, processing of imported pS to mature S within the chloroplast is known to occur in two steps^{23,24}, generating an intermediate which retains a carboxyl-terminal portion of the transit peptide. It is therefore conceivable that this retained portion of the intermediate functions in the recognition of some site in the large subunit to initiate displacement of the large subunit-binding protein and the formation of the holoenzyme complex and that processing of the intermediate to the mature form of S would proceed only after these events have been initiated. Of course, it is also possible that the observed interaction of the anti-idiotypic antiserum with the large subunit of RuBPCase is entirely nonspecific.

All of the 30K immunoreactive polypeptide found in the

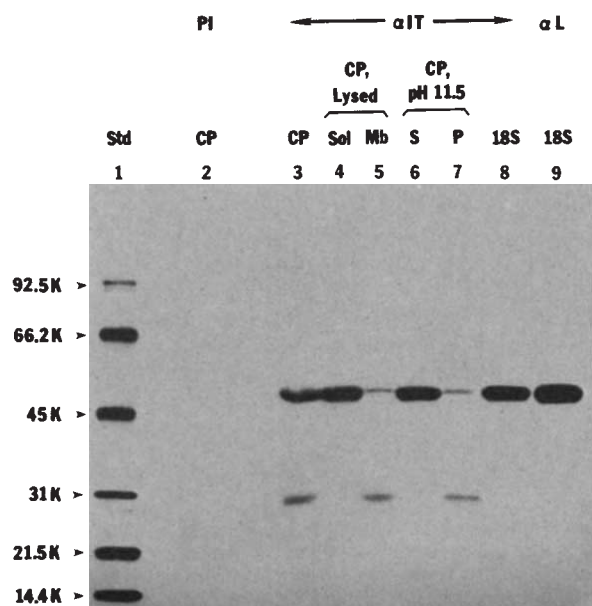


Fig. 5 Identification and characterization of chloroplast polypeptides reactive with anti-idiotypic antibodies. Polypeptides of either whole chloroplasts or of chloroplast subfractions were separated by SDS-PAGE, transferred directly onto nitrocellulose and subsequently probed with different antisera. Lane 1, ^{14}C M_r standards. Lanes 2, 3, entire chloroplast polypeptides (10 μg chlorophyll per lane) were probed with preimmune serum (PI) and α -IT, respectively. Lanes 4, 5; supernatant (S) and pellet (P) fractions after lysis of chloroplasts at pH 7.5, probed with α -IT. Lanes 6, 7; supernatant (S) and pellet (P) fractions of chloroplasts pretreated with alkali (pH 11.5), probed with α -IT. Lanes 8, 9; purified 18S holoenzyme complex of RuBPCase probed with α -IT and antibody against large subunit of RuBPCase (α -L), respectively.

Methods. To isolate the total membrane fraction, chloroplasts (20 μl) equivalent to 40 μg chlorophyll were added to 920 μl of 20 mM HEPES/KOH, pH 7.7, 5 mM EDTA, 1 mM *p*-aminobenzamidine, 5 mM ϵ -aminocaproic acid, 50 units ml^{-1} (trasyolol and 1 mM PMSF, vigorously mixed and incubated on ice for 15 min. NaCl (60 μl , 4 M) was then added and mixed. The lysed chloroplast suspension was centrifuged at 356,000g for 15 min using the Beckman TLA-100.2 fixed angle rotor. The pellet containing the total membrane fraction was dissolved directly in the loading buffer for SDS-PAGE²⁷ and a quarter of the final volume was used per lane for analysis. An equivalent aliquot of the supernatant (250 μl) was precipitated with trichloroacetic acid and processed for SDS-PAGE. For alkali extraction, chloroplasts (40 μg chlorophyll) were treated with 0.1 M Na_2CO_3 , pH 11.5 in a final volume of 1 ml containing 5 mM EDTA, 1 mM *p*-aminobenzamidine, 5 mM ϵ -aminocaproic acid, 50 units ml^{-1} trasyolol and 1 mM PMSF. The sample was vigorously mixed, incubated on ice for 15 min, centrifuged, and pellet and supernatant processed for SDS-PAGE as above. The 18S holoenzyme complex of RuBPCase was purified²¹ and 12 μg per lane was used for analysis. Following SDS-PAGE on 7.5–15% acrylamide gradient gel, proteins were electrophoretically blotted onto nitrocellulose in 20 mM Tris base, 150 mM glycine, 20% methanol and 0.05% SDS at 70 V for 6–7 h at 4°C. Blots were incubated with 3% BSA in 20 mM sodium phosphate pH 7.5, 140 mM NaCl (PBS) for 12 h at 4°C. Subsequent treatments at room temperature unless otherwise stated. Blots were washed with PBS and incubated with PI (1:1,000), α -IT (1:1,000) or anti-large subunit (α -L, 1:2,500) in PBS–1% BSA for 2–4 h. After incubation blots were washed with buffer A (PBS, 0.1% Triton X-100, 0.02% SDS) containing 0.1% BSA and incubated with ^{125}I -labelled protein A (9 μCi μg^{-1} , 118 μCi ml^{-1} , NEN) diluted 1:2,500 in buffer A+1% BSA for 2 h. The blots were extensively washed with 20 mM sodium phosphate pH 7.5, 0.5 M NaCl, 0.1% BSA, 0.5% Triton X-100, 0.1% SDS, dried and exposed to Fuji X-ray film for 6–14 h at -80°C . A separate dried blot containing ^{14}C molecular mass standards was briefly dipped into a solution of 10% PPO (2,5-diphenyloxazole) in toluene, dried and processed as above.

chloroplast (lane 3) subfractionated with the membrane fraction (lane 5), as expected of a candidate for a membrane-bound import receptor. The 30K polypeptide was found to be an integral membrane protein, as it was not extractable from chloroplasts at pH 11.5 (lanes 6 and 7). If the immunoreactive 30K polypeptide is the import receptor, then its SDS-denatured form must have been sufficiently renatured on the nitrocellulose blot, following removal of SDS, so that the conformation of the ligand-binding site and consequently reactivity with the anti-idiotypic antibodies was restored. Precedents for this have been described^{16,25,26}.

To avoid denaturation of the import receptor by SDS altogether, we isolated an import receptor-enriched chloroplast envelope fraction, solubilized the membrane proteins with non-ionic detergent and high salt, radio-iodinated them, and carried out immunoprecipitation with preimmune serum or anti-idiotypic antiserum (Fig. 6). For the isolation of the import receptor-enriched envelope fraction pea chloroplasts were first incubated with nigericin, then with a postribosomal supernatant of a wheat germ cell-free translation mixture containing newly synthesized [^{35}S]Met-labelled pea pS. Because of preincubation with nigericin, pS binds to the chloroplast but is not translocated^{12,27}. Chloroplast-bound pS was separated from unbound pS by centrifugation. Chloroplasts were then disrupted by homogenization and the membrane fraction separated by differential centrifugation. The membrane fraction was resuspended in dense sucrose and subfractionated by flotation into a sucrose gradient. Fractions were collected and aliquots analysed by SDS-PAGE followed by either silver staining (Fig. 6a) or fluorography (Fig. 6b) of the gels.

The thylakoid membranes containing all the chlorophyll (and perhaps some of the inner envelope membrane) remained in the bottom load zone (fractions 18–25) separated from those chloroplast envelope membranes which float into the gradient above (fractions 1–17). The floated chloroplast envelope membranes peaked in gradient fractions 8–11 and contained a polypeptide pattern (Fig. 6a) distinct from that of the thylakoid membrane fraction (fraction 18–25). Note that a 30K polypeptide was the major silver-stained polypeptide of the chloroplast envelope fraction. Most of the import receptor-bound pS (Fig. 6b) was found to float out of the load zone and to coequilibrate with the envelope fraction. In fact, the peak fractions of receptor-bound pS (fractions 8–11, Fig. 6b) cofractionated with the peak fractions of the major 30K polypeptide of the envelope fraction (Fig. 6a).

The envelope peak fraction enriched in import receptor was solubilized with Triton X-100/0.5 M NaCl, radio-iodinated (Fig. 6c, lane 2) and subjected to immunoprecipitation with increasing amounts of preimmune serum (Fig. 6c, lanes 3–6) or increasing equivalent amounts of anti-idiotypic antiserum (Fig. 6c, lanes 7–10). The major silver-stained 30K polypeptide of the envelope fraction (Fig. 6a) is the major iodinated polypeptide (Fig. 6c, lane 2) and is also the major polypeptide immunoprecipitated specifically and nearly quantitatively by anti-idiotypic antibodies (Fig. 6c, lanes 7–10) but not by preimmune serum (lanes 3–6). Taken together with the data shown in Fig. 5, the immunoreactive 30K polypeptide of the chloroplast envelope fraction therefore appears to be the import receptor. The other nearly quantitatively immunoprecipitated polypeptides (marked with asterisks) in Fig. 6c, lanes 7–10 could be polypeptides associated with the import receptor and not dissociated from it by the Triton X-100/salt solubilization.

Conclusions

We have chemically synthesized a 30 residue-long peptide that represents the carboxyl-terminal half of the transit sequence of pea pS (ref. 17) for import into chloroplasts. Antibodies against this peptide immunoprecipitate specifically pS but not mature S, and anti-idiotypic antibodies raised against these anti-peptide antibodies appear to react with the ligand-binding site of the

Fig. 6 Isolation of an import receptor-enriched chloroplast envelope fraction and identification in this fraction of polypeptides reactive with anti-idiotypic antibodies. Pea chloroplasts containing [³⁵S]Met-labelled pS, bound to the import receptor, were disrupted by homogenization. A total chloroplast membrane fraction was prepared, resuspended in heavy sucrose and subfractionated by flotation into a sucrose gradient. Fractions were collected, analysed by SDS-PAGE and gels were silver stained (a) or fluorographed (b). Sucrose gradient fractions 8–11 were pooled. Membrane proteins were solubilized by Triton X-100/0.5 M NaCl, radio-iodinated and immunoprecipitated with preimmune serum or anti-idiotypic antiserum (c).

Methods. Chloroplasts (equivalent to 6 mg chlorophyll) were preincubated in the dark for 15 min at room temperature to deplete endogenous ATP²⁷, then chilled and incubated with 400 nM nigericin^{12,27} on ice for 15 min in a total volume of 3.75 ml containing 50 mM HEPES/KOH, pH 7.7, 0.33 M sorbitol, 1.5 mM DTT, 5 mM EDTA, 5 mM methionine and 100 µg ml⁻¹ BSA. A postribosomal supernatant of a wheat germ cell-free translation mixture containing newly synthesized [³⁵S]Met-labelled pS²⁷ was then added (100 µl). After incubation for 30 min at room temperature in the dark, chloroplast-bound pS was separated from free pS by loading the reaction mixture onto 25 ml of a cushion containing 50 mM HEPES/KOH, pH 7.7, 0.45 M sorbitol, 5 mM EDTA, 2 mM DTT and centrifuging at 4,000g for 1 min. The chloroplast pellet was washed by resuspending it in 25 ml of 50 mM HEPES/KOH, pH 7.7, 0.33 M sorbitol, 5 mM EDTA, 2 mM DTT, and centrifuging as above. The preparation of a total chloroplast membrane fraction and its subsequent subfractionation was done essentially by a published procedure³⁵. Chloroplasts with bound pS were homogenized in 20 ml of ice-cold buffer (20 mM HEPES/KOH, pH 7.7, 0.25 M KCl, 5 mM EDTA, 1 mM *p*-aminobenzamide, 5 mM *ε*-aminocaproic acid, 50 units ml⁻¹ trasylol and 1 mM PMSF) on ice by 15 strokes in a tight-fitting Dounce homogenizer, and the total membrane fraction was pelleted by centrifugation at 27,000g for 40 min. The pellet was resuspended and homogenized as above in a total volume of 10 ml of the same buffer containing 42% sucrose (w/v). The membrane suspension was transferred to a SW 28 centrifuge tube and a linear sucrose gradient (20–38% w/v, 14 ml each) in the above buffer was layered on top. After centrifugation at 96,000g for 17 h, fractions (1.5 ml) were collected from the top. Aliquots (375 µl for fractions 1–17 and 37.5 µl for fractions 18–25) were diluted with water to 900 µl, proteins were precipitated with 10% trichloroacetic acid and processed for SDS-PAGE. Loading of only $\frac{1}{10}$ for fractions 18–25 relative to fractions 1–17 was necessary to avoid overloading with protein and chlorophyll. Samples (in duplicate) were run on two 12% polyacrylamide gels; one gel was processed for silver-staining using the rapid-Ag-stain kit (ICN) according to the supplier's protocol, the other gel was subjected to fluorography²⁷. In a separate experiment, total chloroplast membranes were subfractionated as above except that the pS-binding step was omitted. After flotation, fractions 8–11 were pooled, diluted to 12.5 ml with 20 mM HEPES/KOH, pH 7.7, 4 mM MgCl₂, and the membranes were pelleted by centrifugation at 200,000g for 90 min, then resuspended in 0.5 ml of 20 mM HEPES/KOH, pH 7.7, 0.25 M sucrose, 50 mM KCl, 4 mM MgCl₂ and stored at -80 °C until further use. For radio-iodination the envelope fraction was treated as follows. NaCl and Triton X-100 were added to the membrane suspension to a final concentration of 0.5 M and 1%, respectively. After incubation on ice for 30 min the sample was centrifuged at 356,000g for 15 min using the Beckman TLA-100.2 fixed angle rotor. The supernatant was radio-iodinated using 0.25 mCi Bolton-Hunter reagent (NEN) following the supplier's protocol. The reaction was quenched with 30 mM glycine in 0.2 M Tris/HCl, pH 7.5, and dialysed overnight against 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 4 mM MgCl₂, 0.25 M sucrose, 1% Triton X-100 with several changes. The dialysate was centrifuged in a Beckman airfuge for 20 min at 30 psi to remove aggregates, if any. An aliquot (0.6 µl) was used for each immunoprecipitation assay essentially as described²⁹ except that pre-treatment with SDS was omitted and the immune reaction was carried out in 25 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100 and 1% trasylol. **a**, Numbers on top indicate fraction numbers starting from top of the gradient. Lanes marked Std contain mol.wt. standards with the *M_r* indicated. Arrows between **a** and **b** align corresponding fractions. **c**, Lane 1, ¹²⁵I-M_r standards; lane 2, radio-iodinated membrane proteins of chloroplast envelope fraction (fractions 8–11 of Fig. 6a) solubilized by Triton X-100/0.5 M NaCl; lanes 3–10, for each lane the same amount of radio-iodinated membrane proteins as in lane 2 were immunoprecipitated with 5, 10, 15 or 20 µl of preimmune serum (PI) (lanes 3–6) or 5, 10, 15 or 20 µl of anti-idiotypic antiserum (α-IT) (lanes 7–10). Minor bands in lanes 7–10 are indicated by asterisks.

import receptor on the surface of the chloroplast. Monovalent Fab fragments of these antibodies inhibit import of *in vitro* synthesized pS into isolated chloroplasts, presumably by competing with the transit sequence for binding to the ligand-binding site of the import receptor.

Immunofluorescence microscopy on isolated pea chloroplasts showed that the import receptor was located in patches on the surface of the chloroplast envelope. Immunoelectron microscopy demonstrated that the import receptor was located in contact zones between the outer and inner chloroplast envelope membranes, confirming the suggestion² that receptors for chloroplast import are located in contact zones. In more detailed proposals²⁸ it has been suggested that the import receptor for chloroplasts (and mitochondria) is a constituent of or associated with a proteinaceous pore in the outer and inner membrane. An outer and inner membrane pore might be connected end to end to form a continuous channel that transiently connects cytoplasm and stroma (matrix) for the purpose of protein import²⁸. Each of the contact zones might contain several such protein transport channels which might constitute the *raison d'être* of the contact zones.

The anti-idiotypic antibodies recognize a 30K protein which appears to be an integral membrane protein. Additional evidence for the 30K polypeptide being the import receptor is based on chloroplast subfractionation and immunoprecipitation with anti-idiotypic antibodies under non-denaturing conditions. Subfractionation of chloroplasts containing receptor-bound precursor showed that a receptor-enriched chloroplast envelope frac-

tion can be separated from the bulk thylakoid membranes and contains a major silver-stained 30K polypeptide. Moreover, solubilization of this fraction with Triton X-100 and high salt, radio-iodination and subsequent immunoprecipitation with the anti-idiotypic antibodies yields specific and nearly quantitative precipitation of the 30K polypeptide, strongly suggesting that it is the import receptor.

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LETTERS TO NATURE

Is supernova 1987A a stripped asymptotic-branch giant in a binary system?

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We propose that the progenitor of supernova 1987A was a previously undetected red star in orbit about a blue supergiant (star 1 in the field of Sk–69 202^{1,2}). The progenitor was the remnant of an asymptotic-branch giant that had lost most of its hydrogen-rich envelope to its blue companion by type C mass transfer³. A detailed evolutionary model strongly supports the feasibility of our proposition. We find that the original mass of the supernova precursor was 10–15 $M_{\odot}$ (unless a large fraction of its mass was ejected from the binary system), and its final mass, just before the supernova event, was 3–6 $M_{\odot}$. The system remained bound, with a new orbital period of 3–10 yr and an eccentricity of 0.1–0.4. Our picture can provide plausible qualitative explanations for several anomalies in the observational properties of this supernova.

Before the supernova event, the field of Sk–69 202 contained three known stars, denoted 1, 2 and 3 in the nomenclature of refs 1 and 2. Star 1, by far the brightest star in the visual, has been classified as a B supergiant^{4,5}, but available photometric data indicate that the light from this field is too red for the intrinsic colours of a star of this spectral type and luminosity class⁶. We have placed limits on the brightness of a heretofore unseen red star (which we call star 4) by means of the following procedure (see also ref. 7). We constructed composite spectra of stars 1 and 2, using the photometric data obtained by Isserstedt⁴ and Rousseau *et al.*⁵ and the empirical colour relations given by Johnson⁶. We allowed a range of B2–B5 in the spectral type of star 1, and we assumed that the spectral type of star 2 is no later than B3 (see ref. 8). To allow further for the uncertainties in the colours, we considered the possibility that the luminosity class of each star is either I or V. We did not explicitly include star 3 in our fits. However, as star 3 is also of early spectral type^{9–11}, it almost certainly cannot be the source of excess red light from the field.

The dominant source of uncertainty in our fits is the amount of reddening in the direction of Sk–69 202. If the reddening is high enough, with $E(B-V) \geq 0.16$ (J. Koornneef, personal communication), the presence of a red star brighter than $V=16$ is not needed to obtain a satisfactory fit to the photometric data^{1,12}. For lower levels of reddening, a red star brighter than $V=16$ is required to obtain consistency with the data, unless the colour

of star 1 is very peculiar for its spectral type¹³. For $E(B-V) \approx 0.12$, a $V=14.5$ red star is consistent with the data; for $E(B-V) \approx 0.08$ (a value typical of the Large Magellanic Cloud (LMC) as a whole¹⁴), even a $V=14$ red star cannot be excluded. If the spectral type and class of the red star is that of an early to middle M supergiant, as we expect in the evolutionary model described below, then its absolute bolometric magnitude could be as low as -6 to -7 , for an assumed bolometric correction of $1.5-2 \text{ mag}^{15}$, an assumed distance modulus of ~ 18.6 , and an assumed V absorption of $\sim 0.4 \text{ mag}$. We have also examined the implications of R and I band photometric data (ref. 16 and V. M. Blanco *et al.*, preprint) and objective prism spectroscopic data (V. M. Blanco *et al.*, preprint) and find that these data do not significantly alter our conclusions. A suggestively luminous red star in the field of Sk–69 202 is thus consistent with the available photometric data, in view of the uncertainties¹⁴ in the amount of reddening. We suggest that such a star was indeed present as a binary companion to star 1 and was the actual presupernova.

This type of binary model can explain, at least qualitatively, many of the anomalous features of SN1987A. The essential feature is the presence of a nearby companion (star 1) in an orbit with an initial separation of $\sim 4 \text{ AU}$ and an initial period of $\sim 2 \text{ yr}$. The companion strongly influences the evolution of the supernova progenitor by accreting much of its hydrogen-rich envelope via type C mass transfer³. (Before SN1987A, an asymptotic-branch giant was, in fact, the generally preferred theoretical model for a type II presupernova.^{17,18}) We invoke type C transfer because it allows star 4 to reach the asymptotic giant branch before mass transfer begins, but we cannot exclude alternative models in which mass transfer commences earlier. A reduction of the envelope mass to $1-3 M_{\odot}$ before the supernova event would have lowered the total mass of ejected material and may have thereby reduced the peak visual luminosity of the supernova, while preserving the presence of Balmer lines in the supernova spectrum. A lower envelope mass would also have increased the initial velocities imparted to the ejecta, and higher expansion velocities, would, in turn, have produced a more rapid evolution of the ultraviolet light curve; these inferences also agree with the observational data^{19–23}. Moreover, the occurrence of the supernova in a close binary would provide a plausible explanation for the substantial asymmetries in the expansion of the supernova shell that have been inferred from spectroscopic observations^{24–27}. Although the rapid rise time of the optical light curve has been advanced²⁸ as an argument that the radius of the presupernova was at least a factor of ~ 4 smaller than in our models ($\sim 2 \times 10^{13} \text{ cm}$), the models published by Woosley *et al.*²⁸ suggest that the dependence of the rise time upon the presupernova radius should actually be rather weak. Another advantage of our model is that we have to neither assume a metallicity for the presupernova (ref. 29 and W. D. Arnett, preprint) that is substantially lower than that of most

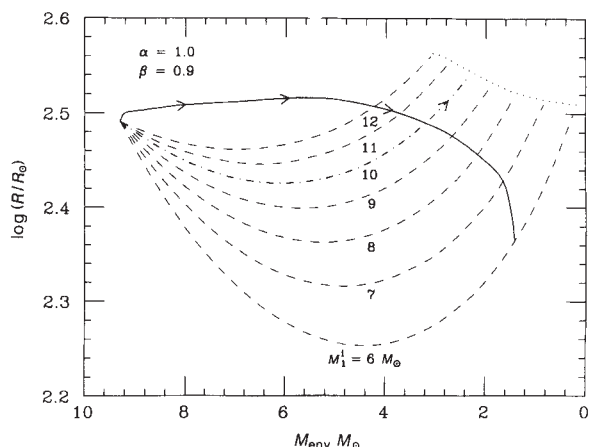


Fig. 1 Solid curve, calculated radius of star 4 as a function of residual mass, M_{env} , for the hydrogen-rich envelope (increasing toward the left), during the dynamical mass-loss phase, for an initial total mass of $12M_{\odot}$ and an initial core mass of $2.7M_{\odot}$. Dashed curves, radius of the Roche lobe of star 4 as a function of M_{env} , for various values of the initial mass, M_1^i , of star 1 and for illustrative values $\alpha = 1$ and $\beta = 0.9$. We assume that the evolution of star 4 follows the solid curve toward the right during the dynamical mass-loss phase, and then follows the appropriate dashed curve toward the right during the subsequent phases of dynamically stable mass transfer. The dot-dashed curve corresponds to $M_1^i = 10M_{\odot}$, and the arrows indicate the evolutionary track followed by our standard model. Dotted curve, maximum radius of star 4 for various values of M_1^i , as indicated, and for other parameter values as stated above.

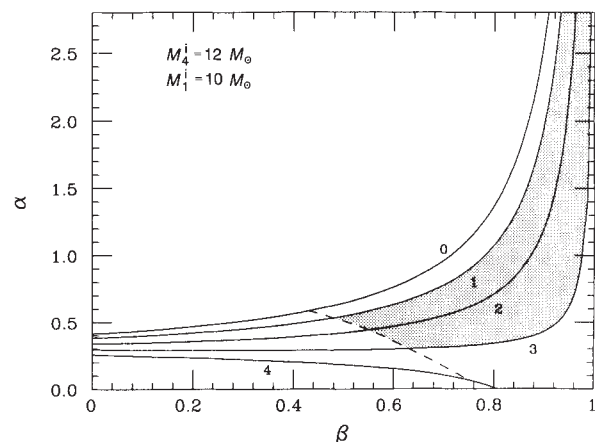


Fig. 2 Solid curves, loci of values of α and β which result in specified values for the final envelope mass, M_{env}^f , of star 4 just before the supernova event. Each curve is labelled with the value of M_{env}^f in solar masses. Dashed curve, locus of values of α and β for which the final mass of star 1 is $14M_{\odot}$, the lower limit allowed by our fit to the photometric observations. (The maximum possible value for the final mass of star 1 in our standard model is $19.3M_{\odot}$, which is substantially lower than the observational upper limit of $\sim 22M_{\odot}$.) Shaded region, domain in α - β parameter space for which $1M_{\odot} \leq M_{\text{env}}^f \leq 3M_{\odot}$ and the final mass of star 1 exceeds $14M_{\odot}$.

early-type stars in the LMC³⁰⁻³², nor introduce a non-standard treatment of convection within the presupernova or a high *ad hoc* mass-loss rate from the presupernova^{28,33}. Irrespective of any specific observational evidence, the fact that a large fraction of all stars occur in binaries^{34,35} makes it worthwhile to determine whether a feasible binary model for SN1987A can be constructed.

For definiteness, we have restricted most of our model calculations to a binary in which the initial primary (presupernova) star has a mass $M_4 = 12M_{\odot}$ and the initial secondary star (now star 1) has a mass $M_1 = 10M_{\odot}$. We have also carried out some additional calculations with the initial value of M_4 as low as $10M_{\odot}$ and as high as $15M_{\odot}$ and with the initial value of M_1 in the range $4M_{\odot} \leq M_1 \leq M_4$. If the initial value of M_4 had been less than $\sim 8M_{\odot}$, star 4 would probably have failed to become a type II supernova³⁶; on the other hand, our calculations indicate that if the initial value of M_4 had exceeded $\sim 15M_{\odot}$, then the presupernova would have been brighter visually than observed. The range of initial masses for star 1 is constrained by the requirement that this star would not have evolved more rapidly than star 4 but could, nevertheless, have become sufficiently massive to be a B supergiant before the supernova event.

Our calculations begin with an approximate numerical model for a $12M_{\odot}$ asymptotic giant-branch star (star 4) with a hydrogen-exhausted core of mass $2.7M_{\odot}$, a metallicity of $Z = 0.02$ in the stellar envelope, and a convective mixing length equal to the pressure scale height. Star 4 is assumed to be in orbit about a $10M_{\odot}$ star (star 1). We do not calculate the detailed evolution of star 1, nor do we attempt to determine the amount of mass loss from either component that may have occurred at earlier evolutionary stages. We choose an initially circular orbit with a separation, a , such that the system would come into contact during the asymptotic giant-branch evolution of star 4, resulting in type C mass transfer³. We use a Henyey-type code³⁷, modified for our present purposes, to follow the subsequent evolution of star 4. The concomitant evolution of the orbital parameters is

followed by the methods developed in refs 38 and 39, and is specified by two free parameters, the fraction β of the mass lost by star 4 that is accreted by star 1 and the specific angular momentum, α , of any matter lost from the system, in units of $2\pi a^2/P$, P being the orbital period. The values of α and β were kept constant throughout each evolutionary calculation. Further details of our theoretical assumptions and computational procedure will be published in a subsequent paper (P.C.J., Ph.P., J.J.L.H. and S.R., in preparation). It is noteworthy that the mass-transfer binary system AZ Cas has measured properties⁴⁰ that resemble in several respects those of the model presupernova system that we propose.

Because M_4 is initially greater than M_1 , the mass transfer, when it commences, is generally unstable and proceeds on a dynamical timescale⁴¹. Our code is unable to treat the hydrodynamics of unstable mass-transfer and we use instead the following approximate technique during this phase (see also ref. 42). During each time step of the evolutionary calculation, we checked to determine whether the radius, R_4 , of star 4 exceeded its Roche lobe, R_L , whose value we determined by application of a formula due to Eggleton⁴³. Whenever $R_4 > R_L$, we removed the outermost $\sim 0.2\%$ of the mass of star 4; the appropriate portions of the removed matter were taken to be accreted by star 1 or ejected from the system, as specified by the prescribed value of β . The remainder of star 4 was then allowed to relax to a state of hydrostatic equilibrium on a timescale that was assumed to be short compared to all relevant timescales except the dynamical timescale for this star. In particular, we assumed that the distribution of specific entropy throughout star 4 was fixed. This procedure neglects the possible hydrodynamic effects of mass motions on the mass-transfer process, but we believe it to be adequate for our purposes.

We discontinued the unstable mass-transfer process when R_4 again became less than R_L . For a specified initial structure for star 4 and a specified evolutionary epoch at which the system comes into contact, the value of R_4 is uniquely determined by the amount of mass that the star has lost. However, the total amount of mass lost from star 4 is a function of α , β and the initial value of M_1 (see Figs 1 and 2). Immediately following the unstable mass-transfer stage, star 4 undergoes an epoch of stable Roche-lobe overflow which proceeds initially on the thermal timescale of the residual stellar envelope and subsequently

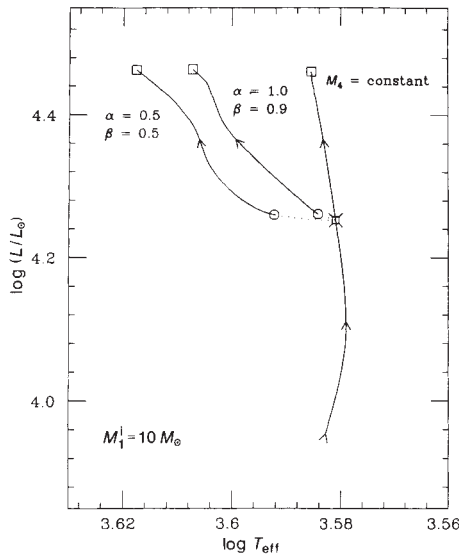


Fig. 3 Evolutionary tracks of star 4 in the Hertzsprung-Russell diagram (stellar luminosity, L , versus effective temperature, T_{eff}), for our standard model and a few illustrative pairs of values of α and β , as indicated. The undisturbed asymptotic-giant branch ($M_4 = 12M_{\odot}$) is shown for comparison. The exploded square indicates the point of departure of the evolutionary tracks from the undisturbed asymptotic-giant branch at the onset of dynamically unstable mass loss; the dotted portions of each curve indicate the phase of dynamical mass loss; the circles denote the onset of the dynamically stable mass-loss stage; the squares indicate the maximum stellar radius (which is achieved at the end of the mass-transfer process in our model calculations); and the arrows denote the sense of the evolution.

on the timescale of nuclear evolution of the stellar core. We follow these evolutionary phases directly using our modified Henyey-type code. We find that for initial values of M_4 less than or of the order of $12M_{\odot}$, this epoch terminates and R_4 begins to decrease before the supernova event; for initial values of M_4 higher than $\sim 15M_{\odot}$, the system remains in contact and R_4 continues to increase until star 4 becomes a supernova. We terminate our evolutionary calculations at or shortly before the final nuclear burning stages that immediately precede the supernova event itself.

The results of our evolutionary calculations are summarized in Figs 1–3. Figure 1 shows the variation of R_4 with residual mass of the hydrogen-rich envelope, for an initial primary mass of $12M_{\odot}$, a range of initial secondary masses, and illustrative values $\alpha = 1$ and $\beta = 0.9$. Figure 2 displays the residual envelope mass, M_{env}^f , of star 4 shortly before the supernova event as a function of α and β . It is evident that there is a substantial swath of plausible values of α and β for which $M_{\text{env}} \approx 1\text{--}3M_{\odot}$, as required in our model. Figure 3 illustrates the evolution of star 4 in the Hertzsprung-Russell diagram, until this star reaches its maximum radius, for our standard model with a few illustrative pairs of values for α and β .

These calculations lend credence to our binary model for SN1987A. We note in particular that the spectral type and luminosity class of star 1 would be consistent with those observed if this star were currently on the main sequence⁴⁴ or in any post-main-sequence evolutionary phase up to the epoch of core helium burning. Any such evolutionary state would, in turn, be consistent with theoretical expectations if star 1 were originally the less massive of the binary components. (However, the thermal readjustment timescale for the envelope of star 1 after the mass-transfer phase is of the same order as the remaining lifetime of the primary, so the luminosity and spectral class of star 1 may have been significantly altered by the preceding mass-accretion episode at the time of the supernova event.) We conclude that a substantial range of values of α and β lead not

only to acceptable values for M_{env} , but also to final masses for star 1 in the range of $18 \pm 4M_{\odot}$, as suggested by our fits to the photometric data for the presupernova. Furthermore, in our standard model, star 4 just before the supernova event is an underluminous, early to middle M supergiant with a V magnitude of 14 ± 0.5 . These theoretical results are consistent with our analysis of the constraints imposed by the available photometric and spectroscopic data for the presupernova field.

In our models, the final mass of star 4 (just before the supernova event) was $3\text{--}6M_{\odot}$. From simple dynamical considerations, we find that a spherically symmetric supernova event should have left the system in a bound orbit, with a new orbital period of 3–10 yr and an eccentricity of 0.1–0.4.

We predict that if our model is correct, star 1 will reappear inside the thinning supernova shell within the next few years. Finally, we note that if the supernova has left a pulsar remnant, it will become straightforward to confirm or refute the duplicity of SN1987A and to verify the orbital parameter values that we have suggested.

After this work was complete we became aware of an alternative binary model for SN1987A proposed by Fabian *et al.*⁴⁵. Features common to their model and ours are that the progenitor of SN1987A was a hitherto unknown binary companion of star 1, that star 1 was not destroyed in the supernova explosion, and that star 1 will reappear after the supernova shell has thinned sufficiently. However, the two models differ dramatically in almost all other respects. The model of Fabian *et al.* invokes a system with the following properties: (1) a progenitor that has evolved only to the first red-giant branch at the onset of mass transfer; (2) pre- and post-supernova orbital periods of ~ 20 days and 50–200 days, respectively; (3) the transfer of the entire envelope of the progenitor to the companion star, leaving only a helium core to evolve to supernova⁴⁶; and (4) the stripped envelope of star 1 as the origin of the hydrogen observed in the supernova ejecta. In contrast, our model has an asymptotic-branch giant progenitor (which was the preferred model for type II supernovae before SN1987A), pre- and post-supernova orbital periods of ~ 2 yr and 3–10 yr, respectively, and a substantial ($\geq 1M_{\odot}$) hydrogen-rich envelope at the time of the supernova. It will ultimately be straightforward to distinguish between these two models.

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Is the nucleus of comet Halley a low density body?

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An important finding of the Vega and Giotto missions to comet Halley was evidence that the cometary nucleus is a consolidated body¹⁻⁵. The shape, size and rotation of the nucleus were studied as well its albedo¹⁻³ and surface temperature⁶⁻⁸. In determining the nature of the cometary nucleus, a value for the average density $\bar{\rho}$ would be informative. In this paper we estimate $\bar{\rho}$ to be $0.6^{+0.9}_{-0.4} \text{ g cm}^{-3}$ compatible with the 'snow-ball' models of the nucleus, although a more compact ice structure could correspond to such a density value.

To find the density we need the mass and the volume of the nucleus. The volume was evaluated by analysis of the imaging data from the Vega-1 and Vega-2 probes², so the problem reduces to the determination of the mass. The traditional way to do this is to measure the gravitational effect of the studied celestial body on the motion of some other body. In this case it is impossible because the mass of the cometary nucleus is too small. Whipple⁹ was the first to propose a unique way to measure a comet's mass. The flow of the substance ejected by the nucleus creates a force influencing the cometary orbit. So in one sense the comet is like a rocket and the mass of its nucleus can be determined if the force and acceleration are known. With this in view, attempts have been made to find the mass of the comet Halley¹⁰⁻¹², but too little was known about the true properties of the nongravitational force before the last apparition of Halley. Now the situation has considerably improved. Rickman¹³ first reported a determination of the mass evaluated using new data, but he took into account only the International Ultraviolet Explorer (IUE) measurements. In this report, on the basis of more detailed and broad observational data, we come to somewhat different conclusions.

Presence of the 'jet propulsion' force provides a systematic difference $\Delta P = 4.1$ days between the observed and 'gravitationally' predicted time of the perihelion passage of comet Halley¹⁴. This force is equal to

$$F = K\dot{M}\bar{u} \quad (1)$$

where $\dot{M}$ is the mass production, $\bar{u}$ is the velocity of flow, K is the factor describing the degree of asymmetry of the flow ($K = 0$ for a spherically symmetrical, $K = 1$ for one-dimensional flow). Let us suppose that vector F lies in the orbital plane (Fig.

1). Then the orbital integral of energy leads to a simple relation for the mass:

$$M = \frac{3aP}{\mu_{\odot}\Delta P} \int_{t_1}^{t_2} K\dot{M}\bar{u}v \cos(\beta - \alpha) dt \quad (2)$$

where a is the semimajor axis, P is the orbital period, $\mu_{\odot}$ is the solar gravitational constant, v is the absolute value of the velocity of the comet, β is the angle between $\bar{v}$ and the Sun-comet direction, α is the angle between the direction of the flow and the comet-Sun direction (Fig. 1), t_1 and t_2 are the times of the cometary activity turn-on and cut-off, respectively. Equation (2) is equivalent to equation (7) of Rickman's work¹³. Its derivation is given in Fig. 1 legend.

We examine below what is known about $\dot{M}$, $\bar{u}$, K and α . We construct three different combinations of these values—nominal (giving the nominal value for the mass of the nucleus), maximal, and minimal models. The latter two will define the limits of uncertainty of the mass evaluation.

Mass production $\dot{M}$. Equations (1) and (2) could be considered as oversimplified at first glance, because a real mass flow involves different components with different values of K , $\dot{M}$, $\bar{u}$ and α . However, the dust velocity near the surface is practically zero, because the dust particles need time to be accelerated by the gas. Thus, we can take into account only the gas. The main constituent of the cometary gas is H_2O . The mass production for the gas is equal to

$$\dot{M}_g = Q_{\text{H}_2\text{O}} m_{\text{H}} \left(\mu_{\text{H}_2\text{O}} + \sum \frac{Q_i \mu_i}{Q_{\text{H}_2\text{O}}} \right) \quad (3)$$

where m_{H} is the mass of the hydrogen atom, $Q_{\text{H}_2\text{O}}$ is the water molecule production rate (s^{-1}), Q_i is the same for the other constituents, μ_i are molecular weights. We will neglect all minor constituents except CO and CO_2 and assume $Q_{\text{CO}}/Q_{\text{H}_2\text{O}} = 0.1$ (ref. 15), $Q_{\text{CO}_2}/Q_{\text{H}_2\text{O}} = 0.015$ (ref. 16).

The water production rate $Q_{\text{H}_2\text{O}}$ depends on the heliocentric distance and this dependence is different before and after perihelion. There are sources¹⁶⁻²⁴ of the observational data giving immediately $Q_{\text{H}_2\text{O}}$ or Q_{OH} and Q_{H} . We assumed $Q_{\text{H}_2\text{O}} = 1/2 Q_{\text{H}}$ in the last case. Figure 2 shows all these data. There is a considerable scatter. This scatter could be explained partially by the rotation and the activity variations of the nucleus. However, there are also some systematic discrepancies between the different sets of the measurements. We will use averaged IUE¹⁷, Suisei²¹, Vega-1 and 2 (refs 16, 18), Giotto¹⁹ and Astron²⁴ data for the nominal model; for the maximal model those from Suisei, Vega-1, Vega-2 and Giotto; and for the minimal only

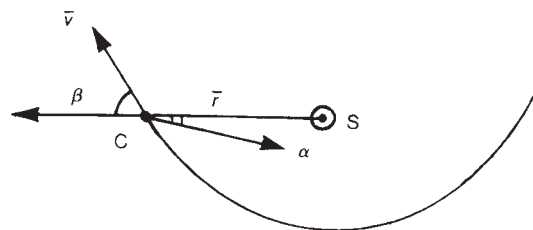
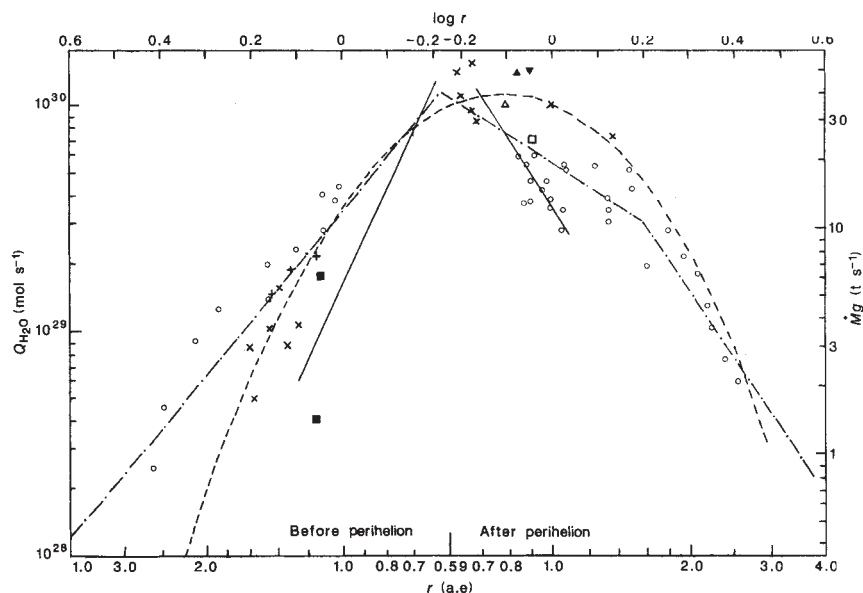


Fig. 1 Definition of the angles α and β , see equation (2). There is a simple relation between variation of the velocity δv shown in equation (2) and the period δP on the osculating orbit: $\delta P = (3vaP/\mu_{\odot}) \delta v$. It can be easily found from the orbital integral of energy $v^2 = (2\mu_{\odot}/r) - (\mu_{\odot}/a)$ and Kepler's third law $P = 2\pi \sqrt{(a^3/\mu_{\odot})}$. r is the distance to the Sun. The angle between vectors $\bar{F}$ and $\bar{v}$ is equal to $\beta - \alpha$, so from equation (1) we obtain for the variation δv during the small time interval dt , $\delta v = (K/\dot{M}) \mu_{\odot} \cos(\beta - \alpha) dt$. The corresponding variation of the period is equal to $\delta P = (3aP)/(\mu_{\odot}\dot{M}) / K\dot{M}\bar{u}v \cos(\beta - \alpha) dt$. Integration of this function on all parts of the orbit where the non-gravitational force must be taken into account leads immediately to equation (2).

Fig. 2 Water molecule production $Q_{\text{H}_2\text{O}}$ and mass production $\dot{M}$ in the comet Halley. x axis — log of heliocentric distance r . $\circ$, IUE¹⁷; $\times$, Suisei²¹; — Dynamics Explorer-1²² ($\equiv$ minimal model B, see text); $\triangle$, Vega-1¹⁶; $\blacktriangle$, Vega-2¹⁸; $\square$, Giotto¹⁹; $\blacksquare$, Kuiper aircraft infrared observatory²⁰; ∇ , rocket L_α photometry²³; $+$, Soviet ultraviolet orbital telescope; $\blacktriangledown$, Astron²⁴; - - - model A (nominal); - - - model B (maximal) of the heliocentric dependence of $\dot{M}_\odot$



Dynamics Explorer-1 (ref. 22) data, though the latter data set is very different from the others.

Velocity $\bar{u}$. Experimental data on gas velocity are available, but not for the region near the surface of the nucleus. Theory predicts that the gas is accelerated in the coma after leaving the nucleus, and the velocity at boundary $\bar{u}$ mentioned in equations (1) and (2) can be evaluated only theoretically. The flux of molecules from unit area due to sublimation in vacuum is:

$$q = n \int_{\Omega_{2\pi}} f(u) u \cos \varphi \, du \, d\Omega = \frac{1}{2} n \bar{u}_{\text{Ox}} = \frac{P_s(T)}{\sqrt{2\pi m k T}} \quad (4)$$

where n is the number density near the surface, $f(u)$ is the Maxwell velocity distribution, φ is the angle between the velocity vector of the individual molecule and the normal to the surface, P_s is the saturation pressure, T is the surface temperature,

$$\bar{u}_{\text{Ox}} = \sqrt{\frac{2kT}{\pi m}} \quad (5)$$

is the mean velocity for the 'hemispherical' Maxwell distribution. Strelinsky *et al.*²⁵ point out that the effective velocity $\bar{u}$ for equation (1) is not equal to $\bar{u}_{\text{Ox}}$, because it is defined by the relation:

$$\bar{u} = \frac{p}{qm} = \frac{1}{q} \int_{\Omega} \int_{2\pi} f(u) (u \cos \varphi)^2 \, du \, d\Omega = \frac{\pi}{2} \bar{u}_{\text{Ox}} \quad (6)$$

where $p = nkT/2 = P_s/2$ is the momentum flux per unit area. The value $\bar{u}$ is almost equal to the mean square velocity.

The gas production rate is $Q = qS$, where S is the cross-sectional area of the nucleus, normal to the Sun direction. We can evaluate n , T and the mean free path l near the surface using gas production data and equations (4) and (5). For example for $Q = 10^{30} \text{ s}^{-1}$ (near perihelion) $n \approx 10^{14} \text{ cm}^{-3}$, $T \approx 205 \text{ K}$, $l \approx 10 \text{ cm}$.

The mean free path is much less than the size of the nucleus ($\sim 10 \text{ km}$) throughout the range of the gas production rate discussed. Thus the description of the process as evaporation in vacuum is not rigorous in this case. It was shown, however, by gas-dynamical calculations²⁶, that correction for q is small ($\leq 10\%$). Kinetic Monte-Carlo modelling²⁵ confirms this conclusion about q and also shows that the correction in the jet propulsion force is small (factor ~ 1.15). There are other complications, however, which may be more important: the evaporating substance of the nucleus contains, beside water ice, other components; much of its surface is covered by some refractory porous mantle; the gas pressure on the night side is not equal to zero.

Thus we have elected not to make any corrections in the relation between the force and the flux of mass and to determine this value we use the simplest model: clean water ice evaporating in vacuum. Neglecting minor constituents in the $\bar{u}$ calculations we will use $m = m_{\text{H}_2\text{O}}$ in equation (6). The temperature T is determined from the balance of the observed water production rate and the flux estimated by equation (5) with P_s data for ice. This temperature varies within a relatively narrow range $175 < T < 205 \text{ K}$ if $4 > r > 0.6 \text{ AU}$. Thus $\bar{u}$ can be approximated by a linear function of $\lg Q$ (or $\lg \dot{M}$) using regression. Hence

$$\bar{u} = 365 + 13 \log \dot{M} \text{ ms}^{-1} \quad (7)$$

Here $\dot{M}$ is the full mass production rate (including CO and CO₂, see equation (3)) in units of ton s^{-1} .

Asymmetry factor K . Images obtained by the Vega and Giotto probes show that only the illuminated side of the nucleus is the source of the cometary dust. Near-infrared spectrometry on Vega-2 (ref. 18) directly demonstrated also a strong asymmetry of the H₂O molecule flux. $K = \frac{2}{3}$ if the local flow is proportional to the solar illumination (for the spherical shape) and maximal possible value is $K = 1$. For the nominal model we assumed the mean of these two limits ($K = \frac{5}{6}$) and for two other models $K = 1$ and $\frac{2}{3}$.

Angle α . This cannot be zero owing to the rotation of the nucleus and the thermal inertia of its surface layer. Measurements of the infrared thermal emission of the nucleus by Vega-1 lead to the lag of the temperature maximum $\sim 10^\circ$ (ref. 7). However, the effective direction of the gas flow does not necessarily coincide with the thermal maximum. I. A. Yastrzhembsky derived values of α and $\dot{M}$ providing the minimum of r.m.s. deviation of the orbit calculated for our nominal model from the measured one (personal communication). He found $\alpha = 2^\circ$ as the most probable value, although this is a preliminary result which will be improved later in a separate paper. Figure 3 shows the dependence of the mass of the cometary nucleus determined by equation (2) from the angle α . Note that with $\alpha = 0$ the jet propulsion effect would have not influenced on the period in

Table 1 Mass, density, mass loss and time t_0 for three models

Model	Mass (10^{11} ton)	Density (g cm^{-3})	$\Delta M/M$ (relative mass loss, %)	Characteristic time t_0 (yr)
Nom (A)	2.9	0.6	0.14	54,000
Min (B)	1.1	0.2	0.50	15,000
Max (C)	7.6	1.5	0.05	150,000

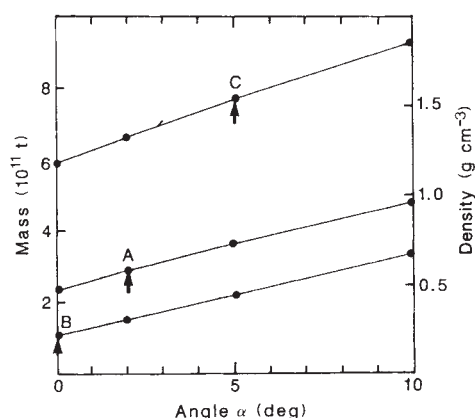


Fig. 3 Sensitivity of the calculated mass and density to the angle α for three models of the mass production. The arrows show the values of this angle assumed by estimations given in Table 1.

case of symmetric shape of $\dot{M}(r)$ function. But $\dot{M}(r)$ values have a pronounced asymmetry and $\Delta P \neq 0$ even with $\alpha = 0$. Asymmetry of $\dot{M}(r)$ explains qualitatively the low value of α obtained by Yastrzhembsky. We suppose that 10° is too large in any case (taking into account asymmetry of $\dot{M}(r)$) and choose $\alpha = 5^\circ$ as the upper limit combining this value with the maximum model of $\dot{M}(r)$. For the minimum model we used $\alpha = 0^\circ$ and nominal 2° .

Values for mass and density calculated for all three models are given in Table 1. The volume is assumed to be $V = 5 \times 10^{11} \text{ m}^3$ corresponding to the ellipsoid $16 \times 18 \times 8 \text{ km}$ used to approximate the irregular shape of the nucleus from Vega images².

The nominal model corresponds to an average density $\rho = 0.6 \text{ g cm}^{-3}$, and approximately three times less or more for the minimal or maximal ones, respectively. Relative mass loss $\Delta M/M$ per revolution is also given in Table 1, with some characteristic time t_0 corresponding to exponential decay of the cometary mass by constant $\Delta M/M$ and P . The real lifetime of the comet is much greater, owing to gradual weakening of the activity and decreasing $\Delta M/M$. There is a possibility that at least some comets 'decay' into asteroids after termination of their activity²⁷.

Rickman¹³ used the same approach but obtained $\rho = 0.1 - 0.2 \text{ g cm}^{-3}$. The main reasons for such a difference are: (1) he used only the IUE data which do not cover the perihelion region and did not take into account that $\dot{M}$ grows considerably in this region according other measurements; (2) he omitted a factor of $\pi/2$ in the expression for the effective velocity. We think that our results are more realistic. An additional, more careful analysis of the gas production data and a better, self-consistent solution for $\dot{M}$ and α may eventually provide a more narrow range for the mass and density evaluation. More precise determination of the volume would also be important for the density estimate. It is also clear that our model of the gas outflow is rather oversimplified.

Even the wide limits of the average density of the nucleus determined here impose some restrictions on its models. These limits are consistent with the published modifications of the 'snow-ball' model of the cometary nucleus^{9,28-32}. For example the nominal value of 0.6 g cm^{-3} is in good agreement with the upper limit (0.7 g cm^{-3}) predicted by Wallis and Macpherson³¹. A more compact ice (mixed with stony material) structure could be acceptable if the density were nearer to the maximum model. However, our evaluations probably permit us to reject potential high-density versions with much stony material such for example as the 'ice-glue' model of Gombosi and Houppis³². A review of the earlier evaluations of density of the cometary nuclei was recently published by Wallis³³. All these previous works (beside Rickman's¹³) were based on some *a priori* models of the composition and structure of cometary nuclei. We have aimed to use

a fundamentally different approach: to evaluate density only from empirical data.

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Equilibrium pressures of oxygen above $\text{YBa}_2\text{Cu}_3\text{O}_{7\pm x}$ up to 2,000 bar

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The interaction of oxygen with the high-temperature superconducting oxides is very important because several phenomena that influence the superconducting properties, such as the orthorhombic to tetragonal phase transformation, are dependent on the oxygen stoichiometry. In spite of this, many of the basic thermodynamic pressure data remain unpublished. The existing information indicates that the decomposition of $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ starts at $T = 400^\circ \text{C}$ at $P_{\text{O}_2} = 1 \text{ bar}$ (ref. 1), and that the compound melts incongruently². Pressure data and corresponding melting points are therefore needed, to ascertain if it is possible to change the phase diagram at higher oxygen pressures so that the compound melts congruently. If this were feasible, then the growth of large single crystals from a stoichiometric melt could be possible. Here we present the equilibrium oxygen pressure curve for the $\text{YBa}_2\text{Cu}_3\text{O}_{7\pm x}$ decomposition (P - T phase diagram) up to 2,200 bar and 1,200 $^\circ \text{C}$. To construct the three-dimensional P - T - x phase diagram of the pseudo-binary system O_2 - $\text{YBa}_2\text{Cu}_3\text{O}_{7\pm x}$, we have also developed methods for measuring the T - x diagram at high pressure, and we present the first data at 10 bar oxygen pressure.

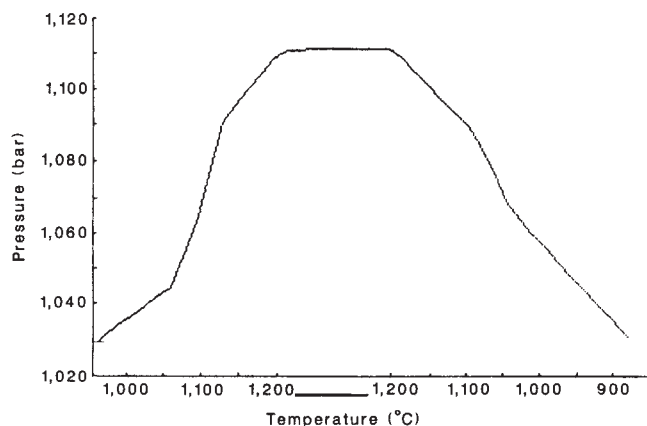


Fig. 1 Increase of pressure as a function of temperature during equilibration of $\text{YBa}_2\text{Cu}_3\text{O}_{7\pm x}$ in a closed crucible. Initial oxygen pressure at room temperature, 630 bar. Note the relative decrease in decomposition pressure after melting ($T = 1,125^\circ\text{C}$ at 1,090 bar).

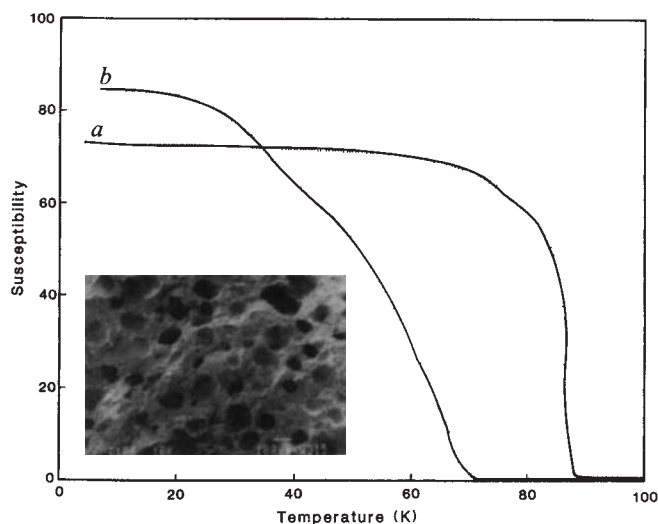


Fig. 2 *a*, Initial susceptibility in arbitrary units as a function of temperature, showing $T_c \approx 70\text{ K}$ for the solidified melt of the experiment shown in Fig. 1, despite decomposition and reaction with the alumina crucible; *b*, the corresponding curve for the starting material. Inset: cross-section of a partly decomposed molten ingot at $1,135^\circ\text{C}$ and 1,095 bar O_2 . This material still has $T_c \approx 70\text{ K}$. Scale bar, $100\text{ }\mu\text{m}$.

The two-chamber high-purity autoclaves used in this work have been described elsewhere³. They are capable of 3,000 bar gas pressure (O_2 , N_2 or H_2) and $T = 1,700^\circ\text{C}$. The main advantage of our pressure system, compared with others described in the literature, is the separation of the atmosphere that is in contact with the sample from the furnace atmosphere. In this way high-purity gas can be used for equilibration with the sample without contamination from isolation and heater. This is accomplished by using a crucible (made from ceramic in the case of oxide samples) to contain the sample. Differential pressure gauges keep the outside argon pressure equal to the inside oxygen pressure.

For the measurement of the equilibrium pressure curve, the crucible was isolated from the reservoirs and remained connected with the pressure transducer only. Initial oxygen pressures in the range 100–1,100 bar were let into the crucible at room temperature and the P - T relationship was measured by slowly increasing the temperature to $1,200^\circ\text{C}$. The maximum

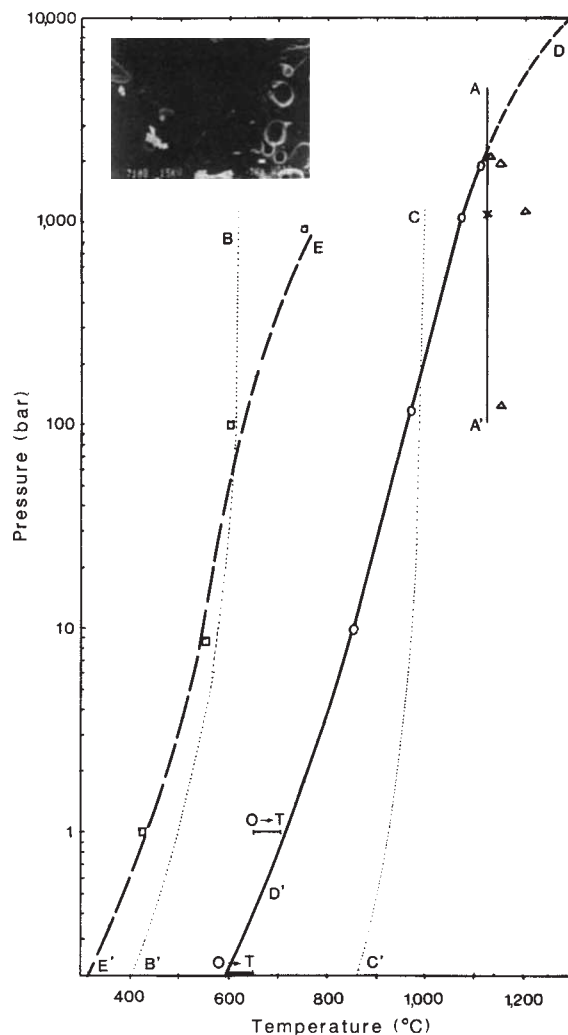


Fig. 3 Oxygen decomposition pressures (up to 2,100 bar) of $\text{YBa}_2\text{Cu}_3\text{O}_{7\pm x}$. Curve D-D' shows the onset of strong decomposition and probably coincides with the orthorhombic to tetragonal transition ('O $\rightarrow$ T' from ref. 1). Curve E-E' shows the onset of the initial slight decomposition, probably overlapping with the $\text{O}_i \rightarrow \text{O}_{ii}$ ref. 5 (for further details see text). Inset: cleared surface of crystal solidified from the melt under 1,975 bar O_2 .

pressure achieved during these experiments was 2,100 bar. A rather unexpected result was that the decomposition takes place in at least two steps. Thus, for example, at 100 bar a slight decomposition starts at 600°C . This slight initial decomposition appears clearly for all samples heated up to 600°C and $P_{\text{O}_2} \leq 100$ bar, and is discussed below. Strong decomposition sets in at 960°C and 115 bar. At $1,150^\circ\text{C}$ this appreciably decomposed sample melted (at 125 bar), as visual inspection after cooling clearly showed.

At 1,045 bar the strong decomposition starts at $1,075^\circ\text{C}$ (see Fig. 1). At $1,125^\circ\text{C}$ and 1,090 bar, the P - T curve exhibits a change in slope, which we attribute to the onset of melting. This leads to a relative decrease in the decomposition pressure, indicating absorption of oxygen by the melt, in agreement with a recently proposed sketch of the T - x phase diagram⁴. After 30 min of annealing at $1,200^\circ\text{C}$, the melt was solidified and cooled at 5°C min^{-1} . Despite the decomposition and reaction with the alumina crucible, part of this multi-phase material was superconducting, with the transition temperature $T_c \approx 70\text{ K}$ (Fig. 2). The Fig. 2 inset shows the morphology of a cross-section of the molten ingot. The bubbles formed as a result of desorption of oxygen from the melt are clearly visible.

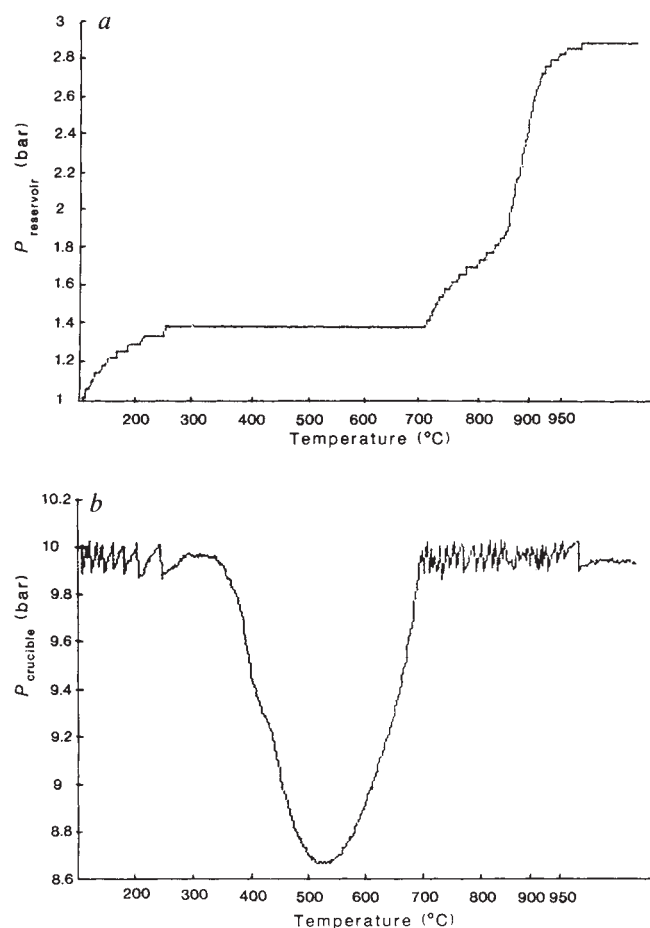


Fig. 4 Pressure changes in the reservoir (a) and above the sample in the crucible (b) as a function of sample temperature. Initial oxygen pressure, 10 bar.

At 1,840 bar O_2 , decomposition starts at 1,110 °C. Heating to 1,150 °C leads to melting. As expected, the solidified material (treated up to 1,975 bar O_2) has many fewer holes than that melted at 1,100 bar, and many monocrystalline areas measuring up to 1–2 mm across.

From this type of experiment can be derived a decomposition curve for $YBa_2Cu_3O_{7\pm x}$ (curve D–D' in Fig. 3). Extrapolation of this and the melting curve (A–A') indicates a melting point at ~1,125 °C, with a decomposition pressure of ~2,400 bar O_2 . If no other irreversible chemical reaction of this compound takes place, then under these conditions one could expect congruent melting and the ability to grow single crystals from the melt. Preliminary X-ray diffraction, energy-dispersive analysis by X-ray (EDAX) analysis and scanning electron microscope data show, however, the appearance of new phases precipitating at higher oxygen pressures. Under the pressure and temperature conditions of the high-temperature side of curve D–D', two phases precipitate as a result of decomposition. EDAX showed that the major precipitate has a composition near Y_2BaCuO_5 , the so-called 'green phase'. Under the assumption of oxygen loss and green-phase formation, a formal decomposition reaction can be written in the form: $2YBa_2Cu_3O_7 \rightarrow Y_2BaCuO_5 + 3BaCuO_2 + 2CuO + \frac{1}{2}O_2$. As yet we have been unable to identify the other solid decomposition products as bulk precipitates.

A further interesting aspect of Fig. 3 is the initial slight decomposition (curve E–E'), which as mentioned above was clearly visible in the P – T curves of samples treated at $P \leq 100$ bar O_2 . As we are not yet able to quench the sample inside the autoclave, we cannot directly investigate possible structural

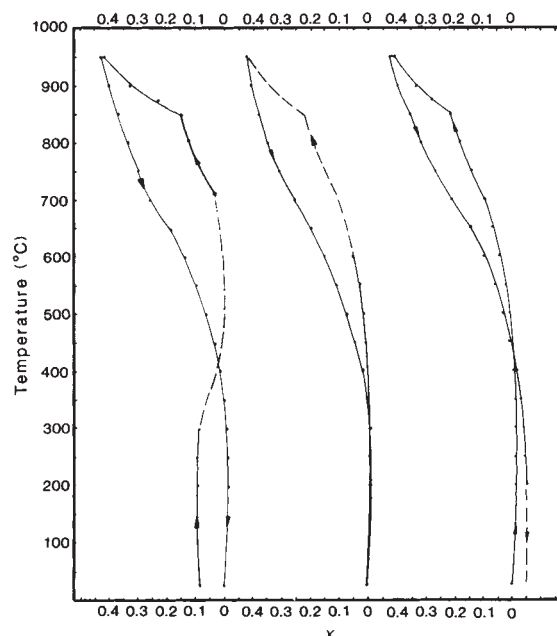


Fig. 5 Temperature-composition (T – x) curves derived from data similar to those shown in Fig. 4, for three consecutive runs with different cooling rates at 10 bar O_2 (run 1 and 2, 5 °C min^{−1}; run 3, 1 °C min^{−1}). x is given by the formula $YBa_2Cu_3O_{7-x}$.

changes following this decrease in oxygen content. But a comparison can be made with the only oxygen-pressure work on $YBa_2Cu_3O_{7-x}$ which we found in the literature⁵. Chen *et al.*⁵ treated samples at oxygen pressures up to 2,000 bar and quenched them from high temperatures; curves B–B' and C–C' in Fig. 3 summarize their results. On the low-temperature side of curve B–B' they found, after quenching, an orthorhombic structure with $a < b \approx c/3$ (O_i); in the field between B–B' and C–C' the structure was also orthorhombic, but with $a < b < c/3$ (O_{ii}); and on the high-temperature side of C–C' the structure was tetragonal, with $a = b < c/3$ (T). As can be seen from Fig. 3, our curve E–E' of the initial slight decomposition almost coincides with curve B–B' of ref. 5. We can therefore assume that this first stage of decomposition does not lead to a change of symmetry but only to the formation of point defects, which nonetheless lead to the disappearance of superconductivity⁵? There is no good agreement, however, between our decomposition curve D–D' and the $O_{ii} \rightarrow T$ transition curve (C–C') of ref. 5. As almost nothing is mentioned about their high-pressure technique, we assume the existence of a systematic error in their measurements and that our decomposition curve (D–D') represents the orthorhombic to tetragonal phase transition resulting from major loss of oxygen. This is in agreement with the $O \rightarrow T$ data of ref. 1 also shown in Fig. 3.

The measurement of the oxygen stoichiometry at high temperature and pressure (T – x or P – x sections of the phase diagram) is very important for the construction of the three-dimensional P – T – x phase diagram, but also experimentally very difficult, if errors arising from the thermal expansion of the gas and the porosity of the sample (at temperatures below the melting point) are to be avoided. To measure changes in the oxygen stoichiometry the pressure in the crucible is kept constant during heating and the excess gas from the decomposition and/or thermal expansion is released by an electronically controlled valve to a calibrated reservoir. During cooling the decrease of pressure from the consumption of oxygen and thermal contraction was compensated by gas added from a calibrated and thermostated reservoir. To enable subtraction of the thermal expansion and contraction effects, calibration runs without sample were performed. In a blank experiment, using

instead of a sample an alumina rod with the same volume, the P - T relationship of the experimental vessel was registered and subsequently subtracted from that of the experiments with real samples. The differences were due to decomposition and/or absorption of oxygen by the sample. In this way, we were able to calculate the oxygen stoichiometry of the samples at high temperatures for an oxygen pressure of 10 bar. All calculations were made under the assumption that the maximum value of x in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ is $x = 0$.

Figure 4a shows the increase in pressure in the reservoir (including that due to thermal expansion) during a heating run of the sample at 10 bar O_2 , and Fig. 4b shows the corresponding changes above the sample (in the crucible). The pressure control is kept at 10 bar. The change in oxygen stoichiometry with temperature for three consecutive runs, calculated from the data of Fig. 4 and the thermal expansion calibration curve, is shown in Fig. 5. In the first run the material absorbs oxygen from 300 to 500 °C, and an almost maximal oxygen content is reached. At 500 °C the initial slight decomposition takes place, leading to a composition $\text{YBa}_2\text{Cu}_3\text{O}_{6.85}$ at 850 °C (see also Fig. 4). At this temperature a much stronger decomposition starts, leading to $\text{YBa}_2\text{Cu}_3\text{O}_{5.5}$ at 950 °C. Cooling at 5°C min^{-1} leads to the maximal oxygen concentration, which we assume to be $x = 0$. The second run shows that stoichiometric material remains stoichiometric after a cycle up to 950 °C. The third run shows that slower cooling (1°C min^{-1}) may slightly increase the oxygen concentration. The change is, however, comparable to the error in our measurement.

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Structural evidence for an isostructural phase transition in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ at the superconducting transition temperature

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There has been intense recent interest in the '1-2-3' family of high-temperature superconductors, including the parent compound $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ (YBCO). This structure¹⁻⁵ consists of corner-linked CuO_4 square-pyramidal groups (nominally Cu^{2+}) connected as sheets in the a - b plane, and distorted square-planar CuO_4 groups (nominally Cu^{3+}) corner-linked parallel to the b direction (see Fig. 1). Although the mechanism of the superconductivity has not been identified, these 'planes' and 'chains' are thought to be involved. The existence of a specific heat anomaly^{6,7} at the transition temperature (T_c) implies a second-order phase transition, with an accompanying discontinuity in the volume expansivity. In addition, Horn *et al.* have inferred from X-ray measurements the existence of a large anomaly in the orthorhombic splitting ($b - a$) close to T_c in YBCO. Here we present precise structural information from neutron powder diffraction indicating an anisotropic isostructural phase transition in YBCO. Unlike Horn *et al.*⁸, however, we do not find a large anomaly in the orthorhombic strain,

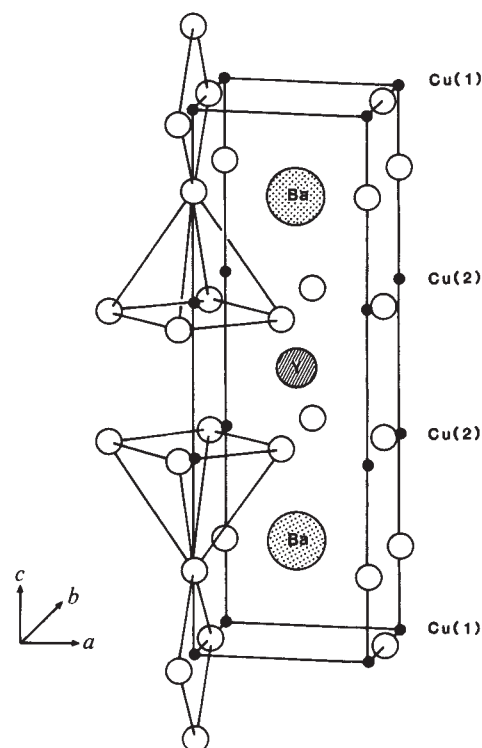


Fig. 1 Structure of $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$.

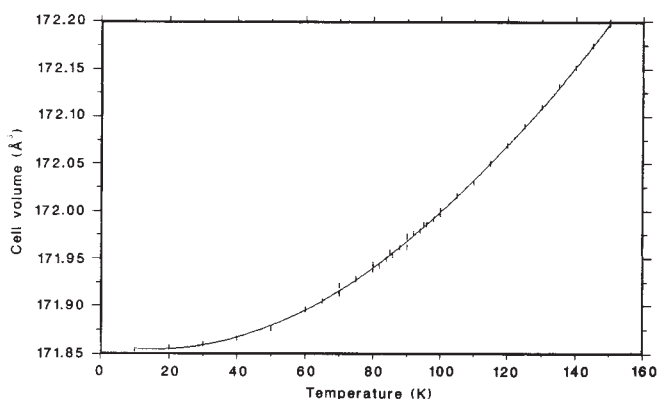


Fig. 2 Volume expansivity in $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$, fit by a double quadratic.

which would seem to contradict their suggestion that the superconducting wave-function is anisotropic in the a - b plane.

A large powder sample of YBCO was prepared from the starting materials Y_2O_3 , BaCO_3 and CuO (each 99.999% pure (Aldrich)). The chemical and phase purity of the starting materials were checked by X-ray powder diffraction ($\text{Cu K}\alpha$ radiation). The powders were thoroughly mixed, ground in an agate mortar and pestle and pressed into pellets, which were fired at 955 °C for 24 h in flowing oxygen, then annealed for a further 6 h at 400 °C, again in flowing oxygen. The electrical and magnetic properties of the sample were then characterized by a.c. inductance and four-point resistivity measurements. The sample showed a superconducting onset transition at 93 K (from resistivity measurements) and a transition width of 2 K. There was no significant residual low-temperature resistivity.

Diffraction measurements were made on the high-resolution powder diffractometer (HRPD)⁹ at the Spallation Neutron

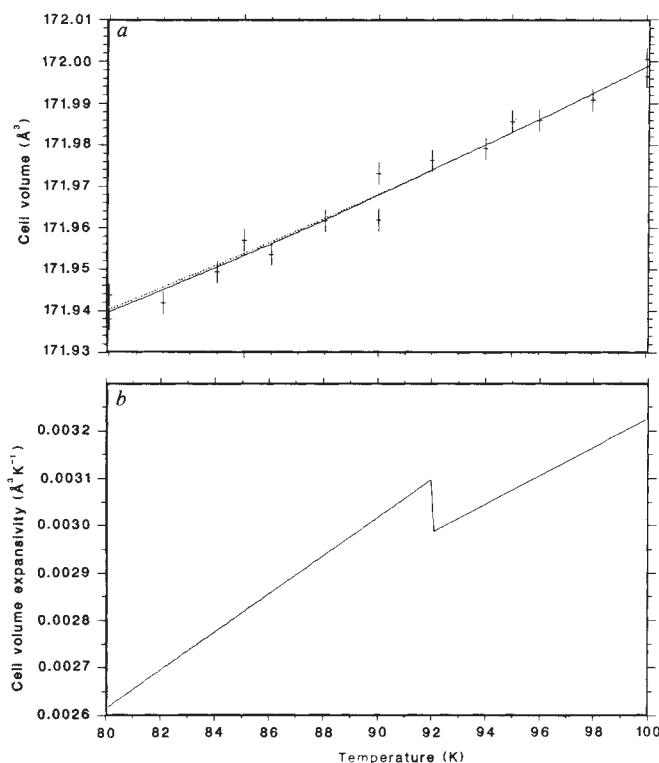


Fig. 3 *a* Volume expansivity fit with extrapolation of normal-state behaviour into the superconducting state, showing the existence of an anomaly. *b*, Plot of the idealized $\partial(\Delta V)/\partial T$ anomaly at T_c .

Source (ISIS), in a liquid-helium cryostat at various temperatures in the range 10–150 K. The overall temperature stability of the sample in the cryostat was <0.1 K. Two passes were made through the superconducting transition temperature of 92 K, the second of these being made after the sample had been removed from and replaced in the cryostat. Measurements made before and after the changing of sample configuration agreed within experimental error. On the second of these runs a 2-K step-scan was made through T_c . For each data set collected, a Rietveld profile refinement was performed, yielding both lattice parameters and atomic coordinates.

In general, for a second-order phase transition, the volume expansivity and specific heat anomalies are related by the following equation:

$$\frac{dT_c}{dp} = \frac{\frac{\partial}{\partial T}(V_s - V_n)_p}{\frac{\partial}{\partial T}(S_s - S_n)_p} = \frac{\left(\frac{\partial}{\partial T}(\Delta V_{sn})\right)_p}{(C_s - C_n)/T_c} \quad (1)$$

that is, the variation of transition temperature with pressure is related to the volume expansivity anomaly divided by the specific heat anomaly.

In two recent experimental determinations of the value of the specific heat anomaly from calorimetric measurements^{6,7}, the values of $\Delta C/T_c$ obtained were $13 \text{ mJ (K}^2 \text{ mol Cu)}^{-1}$ ($56 \pm 28 \text{ mJ K}^{-2} \text{ mol}^{-1}$) and $11 \text{ mJ per K}^2 \text{ mol Cu}$ ($48 \pm 10 \text{ mJ K}^{-2} \text{ mol}^{-1}$), respectively. The value of dT_c/dp in YBCO is small¹⁰, and has been determined¹¹ to have a value of 0.16 K kbar^{-1} , which is supported by thermal expansivity¹², in which the lack of detection of any anomalous change at T_c set an upper limit for dT_c/dp of 0.13 K kbar^{-1} . (This behaviour contrasts with that of the '2-1-4' compounds, $(\text{La, Ba})_2\text{CuO}_4$ and $(\text{La, Sr})_2\text{CuO}_4$, whose large increase in T_c with pressure prompted Chu and co-workers^{13,14} to investigate the Y-Ba-Cu-O system.)

The volume expansivity anomaly was obtained by least-squares-fitting the variation of cell volume with temperature

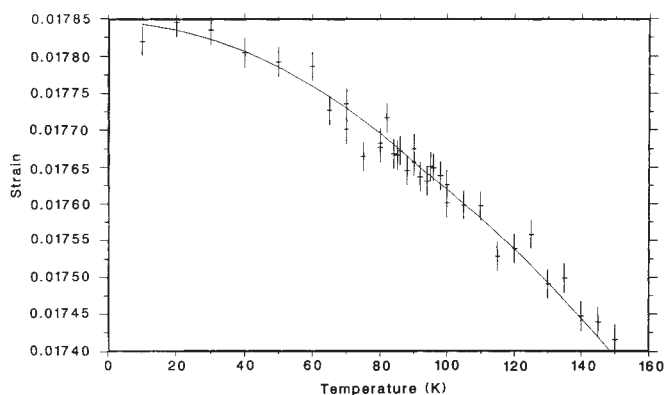


Fig. 4 Orthorhombic strain (defined as $2(b-a)/(b+a)$) as a function of temperature.

(Fig. 2). The cell volume was assumed to vary in a different quadratic manner in the superconducting and normal phases, with the constraint that the cell volume was continuous at T_c . (This constraint is imposed because the transition is not of first order.) The difference between the normal- and superconducting-phase cell-volume behaviour is evident in Fig. 3*a*; the extrapolated normal-phase cell volume does not coincide with the cell volume in the superconducting phase. The derived volume expansivity anomaly, shown in Fig. 3*b*, is found to be $\partial(\Delta V_{sn})/\partial T = (11 \pm 16) \times 10^{-5} \text{ Å}^3 \text{ K}^{-1}$. Using the value of dT_c/dp of 0.16 K kbar^{-1} from ref. 11, this yields a value for the specific heat anomaly of $\Delta C/T_c = 42 \pm 60 \text{ mJ K}^{-2} \text{ mol}^{-1}$. This result is in good agreement with the values of 56 ± 28 and $48 \pm 10 \text{ mJ K}^{-2} \text{ mol}^{-1}$ from the earlier calorimetric measurements^{6,7}, despite the large estimated standard deviation, resulting from peak-shape anomalies associated with the microstructure of the material.

The volume expansivity anomaly implies that there must be an equivalent anomaly in at least one of the lattice parameters of YBCO at T_c . These quantities have been calculated in a similar way to $\partial(\Delta V)/\partial T$ and are: $\partial(\Delta a_{sn})/\partial T = (1 \pm 3) \times 10^{-6}$; $\partial(\Delta b_{sn})/\partial T = (-3 \pm 4) \times 10^{-6}$; $\partial(\Delta c_{sn})/\partial T = (16 \pm 12) \times 10^{-6} \text{ Å K}^{-1}$. As with the volume expansivity anomaly, the estimated standard deviations are large. Consequently, the only conclusion that is tentatively suggested is that the principal component of the volume expansivity anomaly is, perhaps unexpectedly, associated with the c -axis; the a - b -plane dimensions appear to be relatively insensitive to the onset of superconductivity.

This observation prompted an investigation of any possible anomalous behaviour in the atomic fractional coordinates. Assuming different linear variations on either side of T_c , constrained to be equivalent at T_c , led to an indication of anomalous behaviour in the z coordinates of O2 ($1/2, 0, \sim 0.379$), O3 ($0, 1/2, \sim 0.377$), O4 ($0, 0, \sim 0.159$) and Cu2 ($0, 0, \sim 0.356$). The calculated discontinuities are:

$$\begin{aligned} \partial\Delta[z(\text{O2})]/\partial T &= (-3.6 \pm 4.2) \times 10^{-6} \text{ K}^{-1}; \\ \partial\Delta[z(\text{O3})]/\partial T &= (1.9 \pm 4.4) \times 10^{-6} \text{ K}^{-1}; \\ \partial\Delta[z(\text{O4})]/\partial T &= (2.6 \pm 1.8) \times 10^{-6} \text{ K}^{-1}; \\ \partial\Delta[z(\text{Cu2})]/\partial T &= (2.5 \pm 1.5) \times 10^{-6} \text{ K}^{-1} \end{aligned}$$

These values indicate a possible systematic effect in the geometry of the CuO_4 square-planar group and the Cu2–Cu2 separation in the c direction, but also lead to the conclusion that there is no significant anomaly in the length of the apical Cu2–O4 bond associated with the volume and c -axis expansion anomalies.

The experimentally derived quantity $2(b-a)/(b+a)$ can be used as a direct measure of orthorhombic strain in the structure. In contrast to the X-ray results⁸, we see no evidence of substantial anomalous behaviour in this parameter either just below

or even through the superconducting transition¹⁵. The X-ray results showed an upwardly rising cusp from 60 to 80 K, followed by a sharp drop from 90 to 100 K. In contrast, our neutron data (Fig. 4) show a monotonic decrease in the strain parameter, with evidence of at most a very small anomaly. This result was repeatable within experimental error. Thus, our data provide no support for the conjecture that the superconducting wavefunction in this material is anisotropic.

The results presented here further emphasize the differing behaviour of the lattice properties of the 2-1-4 and 1-2-3 superconductors. In the former family there is a definite lattice parameter (spontaneous strain) anomaly with an onset temperature of 75 K, which saturates at 35 K (T_c), consisting of a decrease in lattice distortion and corresponding to a plateau in the d.c. resistivity curve in the same temperature region¹⁶. There is also a large pressure effect on T_c ^{13,14}. That neither phenomenon is exhibited by YBCO has obvious implications for attempts to explain the superconductivity in these compounds. In particular, those theories that seek to describe both types of material in terms of electron-lattice interactions must take account of the fact that the two lattices have widely differing properties at and around T_c .

Neutron scattering facilities for these experiments were provided by the SERC.

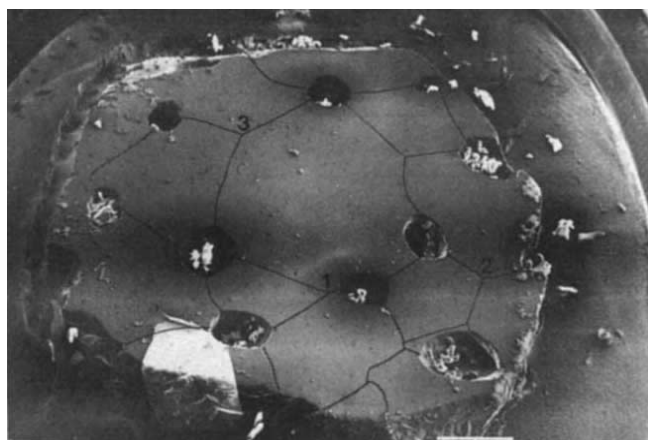


Fig. 1 The ice sample used, showing air bubbles and grain boundaries. A crack is seen running diagonally from top right to bottom left. The black lines have been drawn on to show the location of grain-boundaries, which are seen clearly as grooves on the original photograph. The three triple-junctions where measurements were made are numbered. Scale bar, 1 mm.

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Sulphuric acid at grain boundaries in Antarctic ice

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It has been suggested^{1,2} that acids in the cold polar ice sheets may exist as aqueous mixtures at grain boundaries. This assumption can correctly predict the d.c. conductivity of polar ice², but this does not prove the existence of acids or liquid veins at grain boundaries, and this remains controversial³⁻⁵. In this study we used a scanning electron microscope (SEM), equipped with a cold stage and an energy-dispersive X-ray microanalysis facility, to determine the location of sulphur in ice from the Antarctic Peninsula. As expected, sulphur was undetectable in the bulk of the ice. However, at the junctions where three grains met (triple-junctions), sulphur was found in concentrations greater than 1 M in areas of $<1 \mu\text{m}^2$. Calculations show that between 40 and 100% of the sulphuric acid present in this ice was found at the triple-junctions, and would have been liquid at ice-sheet temperatures. This finding, if general, has considerable implications for many of the physical properties of polar ice.

The ice sample used came from a depth of 65 m (just below the pore close-off depth and with an estimated age of 125 yr), from a core drilled in 1986 at Dolleman Island ($70^\circ 35.2' \text{ S}$, $60^\circ 55' \text{ W}$, elevation 398 m, mean annual temperature -16.75° C) in the Antarctic Peninsula. The samples were transported to, and stored in, the United Kingdom at -20° C ; they experienced their warmest temperature during three weeks storage at the drilling site, where they were buried in a snow pit at a mean temperature of -7° C . No signs of partial melting or recrystallization were observed in the core. The sample was cut from a section whose impurity content, according to measurements by ion-chromatography and atomic-absorption spectrometry, was 182 p.p.b. Na^+ , 320 p.p.b. Cl^- , 764 p.p.b. SO_4^{2-} and 41 p.p.b. NO_3^- . This composition is fairly typical of a summer layer at this site⁶. Sodium can be used to calculate sea-salt content, suggesting that all the Cl^- (9 μM) and 0.47 μM SO_4^{2-} originated as sea salt and that the acid component consists of 7.5 μM H_2SO_4 and 0.7 μM HNO_3 , with no HCl present.

A small slice of the ice was attached to a 10-mm diameter SEM stub. It was then planed to a final thickness of 1-2 mm with successive cuts on a sledge microtome, the final cuts being of 1- μm thickness. This procedure was carried out at -20° C . The ice was transported in a container cooled by dry ice, and finally cooled in liquid nitrogen before insertion onto the cold stage of the electron microscope, as described elsewhere⁷.

The sample was coated with a 23-nm-thick film of aluminium and maintained at $-160 \pm 4^\circ \text{ C}$. Scanning-electron images and X-ray spectra were collected from various areas of the ice using an accelerating voltage of 15 kV and a beam current of 0.5 nA. The X-ray spectra were generally standardized by counting until the aluminium had reached 80,000 pre-set counts. An attempt to calibrate the system using rapidly frozen standards of sulphuric acid in ice was unsuccessful, because these were found to contain substantial inhomogeneities. Therefore concentration estimates are based on measurements of elements (of similar atomic number to S) in gelatine standards⁷. Such estimates may be substantially in error for these samples.

The Antarctic ice was seen to consist of several crystals of average cross-sectional area $\sim 1.8 \text{ mm}^2$ (implying an equivalent spherical diameter of 1.8 mm) with air bubbles $\sim 0.5 \text{ mm}$ in diameter (Fig. 1). Apart from the boundaries, the surface appeared homogeneous even at a magnification of 75,000 implying that there was no substructure at the 10-100 nm scale. A triple-junction seen on the surface would be a linear feature extending into the bulk at some angle to the surface.

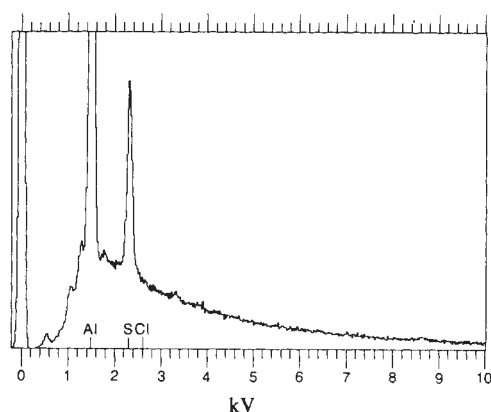


Fig. 2 The spectrum observed in a $1\text{-}\mu\text{m}$ square at the triple-junction denoted by 2 in Fig. 1. The largest peak is from the Al coating.

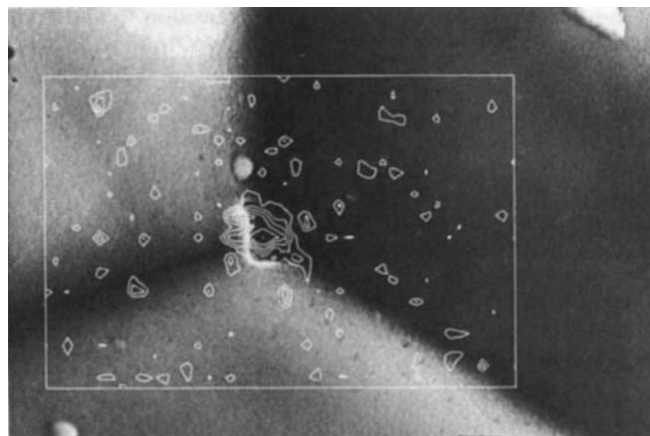


Fig. 3 The area around the triple-junction marked 2 is shown. Superimposed on it is a contour map of the counts received at the X-ray detector for the channels corresponding to sulphur. Counts are registered at the spot where the electron beam entered the sample. The photograph was gridded into squares $0.25 \times 0.25 \mu\text{m}^2$. The mean and standard deviation (SD) of counts in each grid square was calculated. Successive contour lines represent the mean $\pm n$ SD, where n has values of 1–6. The square 'hole' around the triple-junction on the electron image is damage caused by the electron beam when a spectrum was subsequently collected from this small area. Scale bar, $1 \mu\text{m}$.

No sulphur or chlorine was detectable above the background when X-ray spectra were taken from the centre of grains or along two grain boundaries. The detection limit is estimated at 5 mM (that is, 490 p.p.m. H_2SO_4). Areas of $1 \mu\text{m} \times 1 \mu\text{m}$ or $2 \mu\text{m} \times 2 \mu\text{m}$ were probed at three different triple-junctions, and in each case large S peaks were observed (Fig. 2). Chlorine remained below the detection limit. At two triple-junctions an area of $\sim 11 \mu\text{m} \times 9 \mu\text{m}$ was mapped with the detector tuned to S. The result is a map consisting mainly of a uniform field of dots representing the white radiation background, with a greater density of dots around the triple-junction. Because such a diagram does not reproduce clearly, the dot density was contoured. This representation (Fig. 3) shows the extent of the S signal, which covers an area of diameter $1.5 \mu\text{m}$. X-ray excitation volumes are pear-shaped in conductive materials⁸. For materials of low conductivity, such as ice, space-charging will flatten the excited volume⁹. For ice at 15-kV accelerating voltage, the mean excitation depth is $0.8 \mu\text{m}$, and 90% of X-ray emission should occur within $2\text{-}\mu\text{m}$ depth¹⁰. The X-ray resolution will be similar in magnitude. Therefore, if the material in the concentrated area is also of low conductivity, then the pattern of X-rays seen is that expected if the S is actually present in an area of $<1 \mu\text{m}^2$.

The S signal varies greatly between three triple-junctions (with the smallest signal nearly four times less than the largest). Two major reasons may be responsible for most of this variability. First, the count will be dependent on targeting exactly the $1\text{-}\mu\text{m}^2$ area of highest X-ray emission. Second, the angle of the triple-junction to the ice surface is important, because a junction running perpendicular to the surface will have a far greater length within the excited volume than one running nearly parallel to the surface. It is assumed that the triple-junction giving the highest count was well-targeted and perpendicular to the surface, and its count is therefore used in further calculations. This maximum count would suggest a concentration $\sim 1.7 \text{ M}$ in a $1\text{-}\mu\text{m}$ square (but correspondingly higher if the area containing S is actually $<1 \mu\text{m}^2$). In fact, the contour map shows that $>30\%$ of the S counts that could be registered for this triple-junction would come from electrons entering the sample outside the $1\text{-}\mu\text{m}^2$ footprint used in this spectrum. This means that a correction is required to the concentration estimated from uniform standards, suggesting a concentration of $\sim 2.5 \text{ M}$ in $1 \mu\text{m}^2$.

Such a concentration strongly suggests that the sulphur was present as an $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ liquid mixture in equilibrium with ice at the temperatures in the ice sheet. In our experiment, this liquid would have been frozen in place at the eutectic temperature (-73°C) and composition (4.9 M), at which point the area would be $\sim 0.5 \mu\text{m}^2$. But if HNO_3 is also at the triple-junctions, the eutectic H_2SO_4 concentration will be somewhat lower, and the area somewhat larger than this calculation suggests.

Assuming that the triple-junctions form a network of veins $0.5 \mu\text{m}^2$ in cross-section containing 4.9 M sulphuric acid (or its equivalent at larger area and lower concentration), then the meltwater concentration of acid for a crystal dimension of $\sim 1.8 \text{ mm}$ is calculated using a model of polycrystalline ice^{2,11} to be $\sim 7.0 \mu\text{M}$. This number represents 90% of the total sulphate present as sulphuric acid in the sample. It will have an uncertainty of at least a factor two due to various causes, including the use of different elemental standards, and because the veins we measured may not be representative, but it implies that at least 40%, and very possibly all of the H_2SO_4 in the ice is localized at triple-junctions. That any liquid present would exist as veins at triple-junctions, rather than as films at two grain boundaries, was predicted on thermodynamic grounds^{2,11}, but we cannot rule out the presence of some liquid in films, because sulphur in nm-thick films would have been undetectable by this method. The detection limit of this method is far too high for us to make any independent estimate of the concentration of acid within the ice grains.

No chlorine was detected which suggests that $<1\%$ of the Cl in the sample is at triple-junctions. Sodium chloride must therefore be distributed or localized within the grain. We cannot determine whether HNO_3 is also at triple-junctions, because these elements cannot be analysed by this method. The localization of sulphuric acid must be due to processes in the atmosphere or in the ice sheet, and must be related to the far lower eutectic temperature of the sulphuric acid than, for instance, NaCl.

Assuming that this localization is general for Antarctic and other polar ice (in which sulphuric acid is usually at concentrations lower than at this site), it can explain the d.c. conductivity of polar ice². Conduction would be through the network of liquid veins of highly concentrated acid. The veins must occasionally be intersected by air bubbles, so that not all conduction pathways are complete¹² unless there is also a film of acid on the bubbles. Such a film would not be detected by this method. The veins must also be considered in discussion of dielectric properties, particularly at the frequencies relevant to radio-echo sounding of ice sheets. Although the conduction mechanism we describe is plausible, our highly uncertain estimates of the

proportion of acid found in veins do not allow us to rule out the possibility that the remaining acid within the grains may also play a major role.

It is likely that veins of liquid will also have an effect on other physical properties of ice¹³, including its mechanical properties. Finally if similar veins exist in temperate ice, they would provide the mechanism required to explain the early and apparently preferential flushing of acidic impurities from the ice in the spring melt¹⁴. Further studies on samples from other depths and locations are required to establish the importance of the findings reported here.

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Oxygen isotope analyses and deep-sea temperature changes: implications for rates of oceanic mixing

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Since the first measurements were made more than thirty years ago¹, analysis of oxygen isotopes in foraminifera from deep-sea sediments has gained widespread use as a palaeoclimatic and stratigraphic indicator. Isotopes stages are commonly used to define the ice ages² but controversy still remains regarding the relative contributions of temperature and ice-volume change to the down-core $\delta^{18}\text{O}$ record. For the past decade, temperature has commonly been considered a trivial portion of the signal³. Recent reports, however, suggest that as much as one-third of the measured $\delta^{18}\text{O}$ variations can be ascribed to cooler glacial-maximum temperatures^{4,5}. This interpretation requires mean deep-ocean temperatures $<0^\circ\text{C}$ through much of late Quaternary time. Here we explore some of the consequences of this hypothesis for the heat budget of the deep sea, and conclude that either thermohaline overturn must have been faster than at present, or that cooling of the deep ocean at the glacial maximum may have been overestimated.

The oxygen isotope content of calcite is dependent on both the $\delta^{18}\text{O}$ and temperature of ambient water⁶. Thus, down-core isotope analyses must consider both effects. The $\delta^{18}\text{O}$ composition of mean ocean water depends on the amount of ^{18}O -depleted water stored in continental ice sheets. Emiliani¹ measured a glacial-interglacial $\delta^{18}\text{O}$ range of $\sim 1.7\%$ in Caribbean planktonic foraminifera. He ascribed $\sim 0.4\%$ of this signal to ice-volume change, equivalent to 100-m sea-level change with an ice $\delta^{18}\text{O}$ content of -15% (relative to standard mean ocean water, SMOW). This left $\sim 1.3\%$ of the $\delta^{18}\text{O}$ signal to be explained by $\sim 6^\circ\text{C}$ cooling of Caribbean surface waters at the glacial maximum.

Shackleton⁷ demonstrated that $\delta^{18}\text{O}$ variations in benthic foraminifera parallel those of the planktonics. Noting the improbability of identical temperature changes in surface and deep waters, Shackleton argued that ice-volume change dominated the $\delta^{18}\text{O}$ record, and that temperature effects were negligible by comparison. Although arguments about the size of the ice-volume and temperature effects continued⁸, the ice-volume interpretation was supported by the CLIMAP⁹ reconstruction of very small changes in tropical and subtropical sea surface temperatures between the present and the last glacial maximum. Due to these findings, $\delta^{18}\text{O}$ measurements have become a widely used stratigraphic index, assumed to be a globally synchronous record of Quaternary glaciation¹⁰.

A difficulty in this interpretation has been that many studies of sea-level variation based on uplifted coral terraces seem to record a different pattern of variation than is implied by the ice-volume interpretations of $\delta^{18}\text{O}$ (refs 4, 11). A potential explanation of this anomaly is that large floating ice shelves existed at the last glacial maximum, which would contribute to oceanic $\delta^{18}\text{O}$ change, but would not affect sea-level^{12,13}. This hypothesis has been weakened for the Northern Hemisphere by the discovery of continuously fossiliferous sedimentary sequences in the Arctic^{14,15}. It cannot yet be dismissed for the Antarctic, although the proposed shelf averaging 500-m in depth and extending north to 55°S (ref. 13), which would give a global $\delta^{18}\text{O}$ effect of 0.3–0.4%, is generally considered an extreme upper limit.

Most sea-level reconstructions have been made for interstadial high stands^{11,12}. Disagreements in the total range of glacial/interglacial sea-level variations, ranging from 90 m (ref. 16) to 175 m (ref. 17) have precluded a definitive reconstruction of the full sea-level record. Recently, a detailed record of sea-level over the past ~ 200 kyr, including glacial low stands, has been inferred from uplifted terraces in New Guinea¹⁸. Chappell and Shackleton⁴ modify this record slightly and compare it to a high-resolution benthic foraminiferal $\delta^{18}\text{O}$ record from core V19-30 in the eastern tropical Pacific ($3^\circ 21'\text{S}$, $83^\circ 21'\text{W}$, 3,091-m depth). Based on the misfit of the sea-level and $\delta^{18}\text{O}$ curves (and assuming no significant ice-shelf $\delta^{18}\text{O}$ effects), they conclude that the deep Pacific maintained its present temperature of 1.5°C for only brief (~ 10 kyr) periods during interglacial extremes, and that its temperature fell to $<0^\circ\text{C}$ during much of the late Quaternary. In terms of oxygen isotopes, this would mean that the $\sim 1.7\%$ $\delta^{18}\text{O}$ signal is partitioned into an ice-volume effect of 1.3% and a temperature effect of 0.4% at this site.

Analysis of $\delta^{18}\text{O}$ in benthic foraminifera from the deep Norwegian Sea⁵, where modern temperatures are $<-1^\circ\text{C}$, support and extend this interpretation. Temperatures here are constrained by the freezing point of sea water (-1.9°C at the sea surface), and thus cannot contain significant temperature signals. Note that *in situ* temperatures at depth are higher than at the sea surface, due to compression of sea water. This effect is $\sim 0.1^\circ\text{C}$ per 1,000 m depth¹⁹, so the coldest temperature possible at a mean abyssal depth of 2,000 m is -1.7°C . The maximum temperature effect that could possibly be recorded by Norwegian Sea benthics is $\sim 0.5^\circ\text{C}$ or 0.1% $\delta^{18}\text{O}$.

Based on the Norwegian Sea $\delta^{18}\text{O}$ data, Labeyrie *et al.*⁵ infer a glacial/interglacial ice-volume $\delta^{18}\text{O}$ effect of 1.1%. Extending this value to cores in the North Atlantic, Pacific and Antarctic, they hypothesize widespread deep-ocean cooling of 2–3 $^\circ\text{C}$ during glacial time, giving a $\delta^{18}\text{O}$ temperature effect of 0.5%–0.7%. This would require *in situ* temperatures of less than -0.5°C throughout the global oceans below 2,000 m at the glacial maximum.

If correct, this interpretation has significant consequences for glacial deep-ocean circulation. Here we explore the implications for rates of deep-ocean mixing by constructing a simple heat-budget model of the deep ocean. Heat fluxes into the deep sea come primarily from three sources: formation of deep water, geothermal heating, and cross-thermocline diffusion²⁰. At steady

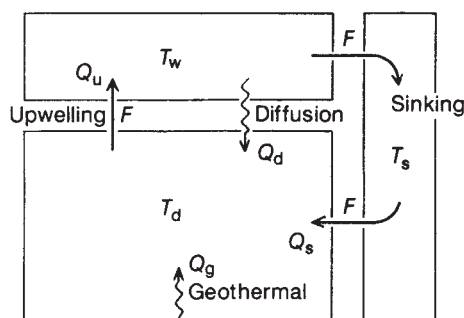


Fig. 1 Schematic heat budget of the deep sea. Heat loss from the deep ocean by upwelling (Q_u) is balanced by heat input from sinking near-surface water (Q_s), geothermal heating (Q_g), and cross-thermocline diffusion (Q_d). The combination of these effects plus compressional heating control the mean *in situ* temperatures of the deep sea.

state, these heat gains must be balanced by upwelling of water to the surface at the same rate as the formation of deep water.

This model is illustrated conceptually in Fig. 1, and expressed quantitatively as follows:

$$FT_d C_p = FT_s C_p + H_f A_b + (T_w - T_b) K_t C_p A_w \quad (1)$$

or

$$\text{upwelling} = \text{downwelling} + \text{geothermal heating} + \text{diffusion}$$

where T_d , T_s and T_w are the temperatures of mean deep water, sinking water and warm surface water, respectively. F is the deep-water formation rate, defined by $F = V_o/\tau$ where V_o is the volume of the ocean, $1.35 \times 10^{24} \text{ cm}^3$ (ref. 21), and τ is the mean age of deep water. C_p is the heat capacity of sea water ($0.94 \text{ cal cm}^{-3} \text{ per } ^\circ\text{C}$), and H_f is the mean geothermal heat flux. A_b and A_w are the areas of the sea floor and the warm surface ocean, here taken as $3.62 \times 10^{18} \text{ cm}^2$ and $2.72 \times 10^{18} \text{ cm}^2$ respectively. K_t is a discrete transfer coefficient²², defined by $K_t = 2K_z/Z$, where K_z is the eddy diffusivity and Z is the mean depth of the ocean, 3,900 m.

We calibrate this model for the modern world, using the observed mean temperatures of 20°C for the warm surface ocean, 0°C for sinking (new) deep water, and 1.7°C for mean deep-water potential temperatures (or 1.9°C *in situ* at 2,000 m)²⁰. Given a thermohaline overturn rate of 43 Sv ($4.3 \times 10^{13} \text{ cm}^3 \text{ s}^{-1}$) appropriate for a mean deep-water age of 1,000 yr (ref. 23), the modern temperature difference between sinking deep water and mean deep water is maintained by geothermal heating of $1.3 \times 10^{-6} \text{ cal cm}^{-2} \text{ s}^{-1}$ (ref. 24) and cross-thermocline diffusion of heat with a vertical diffusivity of $0.3 \text{ cm}^2 \text{ s}^{-1}$. This value for K_z is within the range of most estimates for cross-thermocline heat transfer²⁵. Gordon²⁰ notes that although eddy diffusivities for continuous thermocline models are typically considered to be $\sim 1 \text{ cm}^2 \text{ s}^{-1}$, roughly two-thirds of this heat flux warms water that is immediately upwelled. Thus, for our box model, a diffusivity of $\sim 0.3 \text{ cm}^2 \text{ s}^{-1}$ is appropriate. The effect of cross-thermocline mixing on deep-sea temperatures is roughly 15 times that of geothermal heating.

The effect on mean deep-sea temperature of ventilation rate and sinking water temperature is shown in Fig. 2. It is not possible to attain glacial deep water *in situ* temperatures of -0.5°C , as required by the Labeyrie *et al.*⁵ reconstruction, while maintaining sinking-water temperature at its present mean value of 0°C (Fig. 2, line *a*). Even if mean sinking-water temperatures were reduced to the freezing point of -1.9°C , nearly a doubling of thermohaline overturn would be required (Fig. 2, line *b*). Note that this is a limiting case. A more realistic glacial mean sinking temperature might be -1°C , which would require even

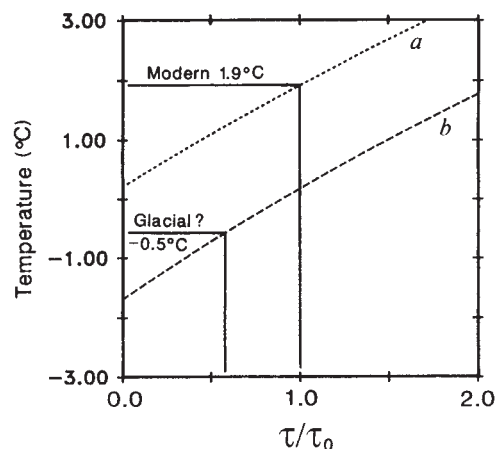


Fig. 2 Deep-sea *in situ* temperatures (at depth 2,000 m) as a function of deep-water ages for box-model ocean. τ/τ_0 is the ratio of the mean age of deep water to that at present. Line *a*: *in situ* temperatures for mean sinking-water temperatures of 0°C (the modern case). Line *b*: same as *a*, but for mean sinking-water temperatures of -1.9°C (the limiting case for the freezing point). A more realistic description of the glacial ocean would lie between *a* and *b*. Mean deep-ocean temperatures of -0.5°C , as reconstructed for the glacial ocean, cannot occur without both significant cooling of mean sinking waters and reduction of mean deep-water ages (increase of thermohaline overturn).

faster ventilation. Alternatively, to achieve mean deep-water temperature of -0.5°C without faster deep-water formation, reduction of cross-thermocline diffusion of heat (perhaps by a 10°C cooling of warm surface waters or a 50% reduction of diffusive mixing) during the glacial maximum would be required.

Are any of these models reasonable descriptions of the glacial ocean? Large ($\sim 10^\circ\text{C}$) cooling of mean warm surface waters appears unlikely. The CLIMAP⁹ reconstruction of the glacial surface ocean, which was based on abundances of fossil plankton species, suggests mean cooling of $<2^\circ\text{C}$. Evidence for mountain glaciation has been used to suggest slightly greater sea surface cooling ($\sim 4^\circ\text{C}$, ref. 26), but $\delta^{18}\text{O}$ data from planktonic foraminifera are consistent with smaller temperature changes²⁷. No reconstructions have suggested glacial cooling of 10°C for the mean surface ocean.

Glacial thermohaline overturn faster by a factor of two than at present also seems unlikely. Benthic-planktonic differences in ^{14}C ages are similar to those at present back to at least 12 kyr BP²⁸ and are equal to or greater than at present during the glacial maximum²⁹. This by itself does not rule out increased rates of overturn, but would require lower ^{14}C content in newly formed deep water during glacial time to offset the faster turnover. That is, glacial North Atlantic Deep Water (NADW) would have to form with low preformed ^{14}C , similar to deep waters formed in the Weddell Sea today. This 'preformed properties' model has been suggested earlier based on stable isotope data³⁰, but refuted based on the inferred low nutrient content of glacial NADW³¹. Further evidence against higher rates of thermohaline overturn in glacial time comes from calcium carbonate budgets. Broecker³² found higher carbonate productivity in interglacial than in glacial time and inferred glacial thermohaline overturn slower than at present. Thus, with available data, ventilation rates faster than at present do not seem likely for the glacial maximum.

Caution is advised in quantitative interpretation of model results. All simple models, including the one constructed here, use eddy diffusion (K_z) to simulate heat transports. While this is a reasonable approach, the true physical processes underlying this effect remain poorly understood. Some of the simplifications

in our study will ultimately need to be addressed in more complex models. For example, intermediate-water formation, which is not explicitly included here, may have varied significantly in glacial time³³. Cooling of intermediate waters enough to cut downward heat transport in half, however, would leave a record of high-amplitude benthic $\delta^{18}\text{O}$ variations in shallow (<1,500-m depth) sediment cores. This has not been observed³⁴.

Thus, although we raise the caveat about applying simple models to complex situations, we believe the conclusion is inescapable that significantly cooler deep-ocean temperatures at the glacial maximum would require increases in rates of thermohaline overturn. In opposition to this, many studies call for reduction of deep-water formation rates at the last glacial maximum³⁵. A possible implication is that cooling of the deep sea has been overestimated by considering benthic $\delta^{18}\text{O}$ records from the Norwegian Sea to be pure ice-volume records⁵. The deep Norwegian Sea may have been slightly fresher (and thus lower in $\delta^{18}\text{O}$) relative to the rest of the ocean at the glacial maximum than it is today. This would allow ice-volume effects on $\delta^{18}\text{O}$ >1.1‰ (say, 1.4–1.6‰), while still permitting more modest changes in mean deep-ocean temperature of perhaps 1–1.5 °C during the late Quaternary. In this case, the misfit of sea-level and $\delta^{18}\text{O}$ data would not be explained, although the existence of large ice shelves that contributed to $\delta^{18}\text{O}$, but not sea-level, remains a possible explanation.

On the other hand, if the ice-volume effect on $\delta^{18}\text{O}$ was as small as 1.1‰, and mean deep water cooled to <0 °C, the mechanisms of glacial deep-water formation and thermohaline circulation must have been radically different than at present to maintain the inferred very cold deep-ocean temperatures.

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Meridional heat transport across the Sub-Tropical Convergence by a warm eddy

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The heat transport of the ocean–atmosphere system is strongly dependent on the meridional transport of heat in the oceans and in particular that between the Southern Ocean and adjacent ocean basins. This ocean process may possibly be dominated by the propagation of energetic mesoscale eddies at certain distinct sectors of the Sub-Tropical Convergence such as those bordering the western boundary currents where eddies could be preferentially spawned. I report here for the first time the shedding of a warm ocean eddy from the Agulhas Current across the Sub-Tropical Convergence, its internal structure as well as rotational frequency. The subsequent drift of the eddy and its further interaction with the Sub-Tropical Convergence is documented by sequential satellite infrared measurements as well as the behaviour of a free-drifting buoy placed in the feature. Based on simultaneous hydrographic measurements, the total heat flux across the Sub-Tropical Convergence achieved by the eddy is estimated at 26.7×10^{17} J.

The meridional heat transport of the world ocean is a crucial element of the global climate system^{1–4}. Global ocean exchange and interaction is achieved primarily through the substantial connection between the individual ocean basins and the circumpolar annulus of the Southern Ocean. Estimates of interbasin and meridional heat flux in the Southern Ocean have relied heavily on historical hydrographic data^{5–7} and inconsistencies and uncertainties in these and other estimates^{8,9} have been ascribed mainly to heat carried by baroclinic ocean eddies. This hypothesis has been substantiated by results of long-term current measurements in the Drake Passage, which have established¹⁰ that the heat flux across the Antarctic Polar Front is dominated by events with timescales of 5–60 days that occur ~3–10 times per year and are most probably related to the spawning of frontal eddies, whereas estimates of intrusive heat flux across the Polar Front due to interleaving between adjacent water masses were minor by comparison¹¹. The production of such heat-transporting eddies has, in fact, been observed in the Southern Ocean, particularly near well-defined ocean fronts^{12–14}. Although the predominant mechanisms responsible for poleward heat flux across the Southern Ocean frontal regions has yet to be identified unambiguously, it is evident that the production rate of mesoscale eddies at these frontal systems may play a critical role⁹.

The northernmost border of the Southern Ocean is the Sub-Tropical Convergence and a recent review¹⁵ has suggested that it may prove most practicable to determine net ocean heat flux into the Southern Ocean here. The intensity of this front, and therefore its potential for high heat flux, as well as the mesoscale eddy turbulence of the front is highly geographically dependent. Results of analyses by historical hydrographic¹⁶, satellite altimetric¹⁷, satellite thermal infrared and drifting-buoy data¹⁸ present a mutually consistent geographic distribution of areas with high variability and high eddy kinetic energy. One of the identified areas of very high variability is the terminal region of the Agulhas Current abutting the Sub-Tropical Convergence. The production of Agulhas rings from the Agulhas Current^{19,20} as well as the spawning of a suite of both cold and warm eddies across the Sub-Tropical Convergence in this area has been identified²¹. With knowledge of the average heat content of these eddies and their production rate an estimate may in principle be made of the meridional heat transport at these high-energy areas, which could be acting as favoured heat conduits into the Southern Ocean.

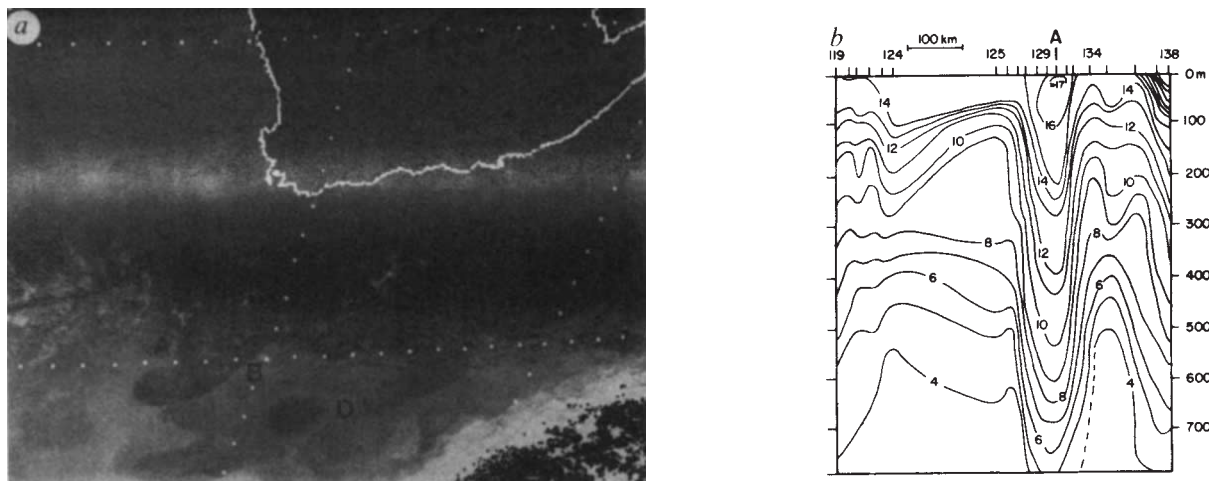


Fig. 1 *a*, Thermal infrared image from the Meteosat II satellite of the ocean region south of Africa for 15 November 1983. Identifiable mesoscale features include the Agulhas retroflection (A), the Sub-Tropical Convergence (STC; B) and an Agulhas ring (C). A discrete and distinct warm eddy is distinguishable south of the STC (D). *b*, Temperature section based on hydrographic stations in the warm eddy (D). Stations correspond to those occupied on the cruise track shown in Fig. 2. The point denoted A corresponds to the most southern point of the cruise track at which there was a change in direction (Fig. 2).

A warm eddy of sub-tropical origin was identified in sub-Antarctic waters south of the Sub-Tropical Convergence and studied in detail for the first time during a multiship exercise in the ocean sector south of Africa from October to December 1983. The eddy was first observed in contemporaneous satellite thermal infrared imagery (Fig. 1*a*), and the cruise track of a participating research vessel, the RV *Knorr*, directed to its centre. Satellite imagery was from the radiometer of Meteosat II measuring in the 10–15 μm part of the spectrum, declouded daily and suitably contrast-enhanced in the appropriate oceanic temperature range.

Standard hydrographic measurements consisting of conductivity-temperature-depth (CTD) probe readings, expendable bathythermography and continuous surface thermography were taken from the RV *Knorr* and a drifting buoy released close to the eddy's centre. The drifting buoy was a 2-m spar buoy with a 2-m² window shade drogue telemetering via the Argos satellite

system. Although the eddy was not traversed completely, simultaneous and subsequent satellite imagery suggest that the feature was only slightly ellipsoidal at this stage and it is assumed that the combined inward and outward thermal sections (Fig. 1*b*) approximate a true cross-section. The eddy exhibited a water mass distinctly different from its direct environment. Temperature/salinity (*T/S*) relations show that its water mass consisted of sub-tropical surface water modified by cooling in the upper 100 m, whereas that of adjacent waters was clearly sub-Antarctic surface water²². Chemical nutrients and trace metal measurements support the conclusion that the eddy is of sub-tropical origin²³. The vertical extent of the eddy exceeded 1,500 m, the depths to which measurements by CTD sensor were made²², and it had an average diameter of ~120 km. Both the characteristics of the eddy mentioned above and its location categorize it as an Agulhas retroflection eddy²¹.

The contrasting sea surface temperatures between the eddy

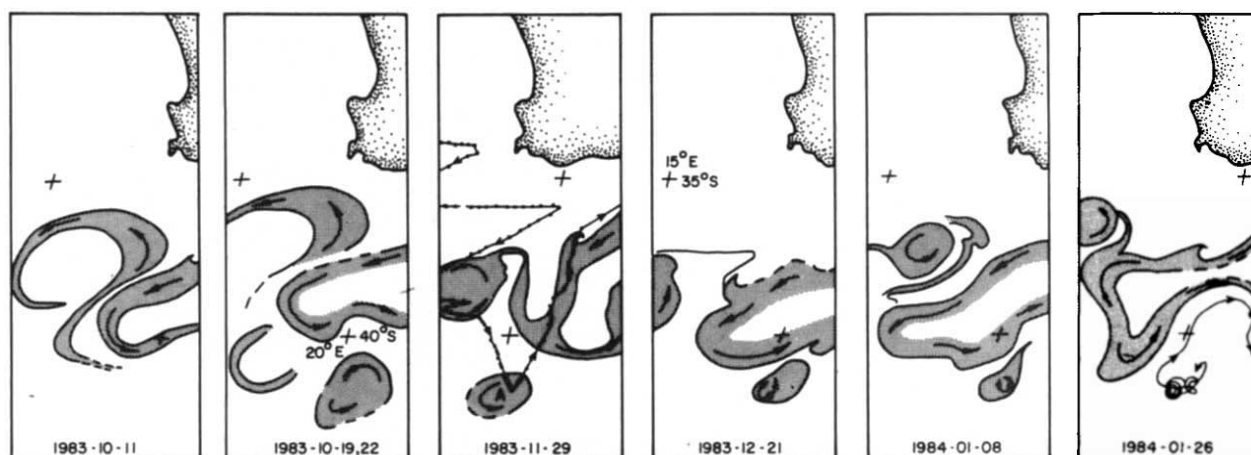
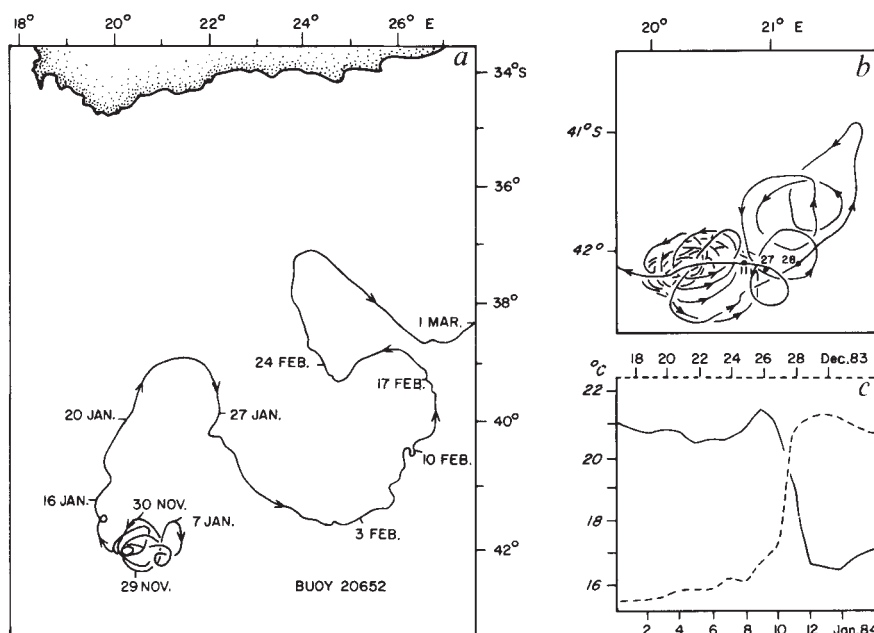


Fig. 2 Portrayal of the shedding of a warm eddy (D, Fig. 1*a*) across the Sub-Tropical Convergence, its gradual shrinking and eventual fragmentation according to thermal infrared imagery from the Meteosat II satellite. The station distribution which is superimposed on the eddy configuration for 29 November 1983 consists of that part of the cruise track of the RV *Knorr* that formed an incursion into the eddy between 26 and 28 November 1983, turning at point A. The temperature section for these stations is shown in Fig. 1*b*. Solid arrows denote inferred flow directions. A dot shows the position of a drifting buoy, placed in the eddy on 27 November 1983, on three subsequent days with parts of its contemporaneous drift track.

Fig. 3 *a*, Drift track of a buoy placed in a warm eddy south of the Sub-Tropical Convergence on 27 November 1983, with detail of the drift track up to 12 January. *b*, Dots show buoy positions on 27 November, 28 December and 11 January 1984. The latter two dates correspond to drastic temperature changes measured by the buoy and shown in (*c*). The broken-line abscissa corresponds to the broken-line temperature curve.



and its environment facilitated its identification on intermittent cloudfree satellite imagery (Fig. 2). Not visible in sea surface temperatures on or before 11 October 1983, it was clearly evident by 19 and 20 October. It may, therefore, be assumed with confidence that it was shed from the Agulhas retroflection during this period. Thermal infrared imagery unambiguously shows the spawning of a distinctly similar warm feature at the Agulhas retroflection from 15 November to 7 December 1983 but no hydrographic measurements could be made of this eddy. The former eddy showed very little translation subsequent to spawning but a consistent lateral reduction in size over 3.5 months. Between 29 November and 8 December the Agulhas retroflection characteristically protruded eastward²⁴ decreasing the distance between the Sub-Tropical Convergence and the eddy to <30 km. The buoy placed in the eddy had carried out nine revolutions about the eddy during this period (Fig. 3*a, b*), with unvarying periods of 3.1 days and average speeds of 65 cm s^{-1} . This speed is comparable with that estimated for water movement in the Agulhas retroflection loop by geostrophic calculation relative to 1,500 dbar of between 107 cm s^{-1} and 50 cm s^{-1} (ref. 22). The volume transport to 1,500 m, calculated for the westernmost section (Fig. 2) was $32 \times 10^6 \text{ m}^3 \text{ s}^{-1}$, about half that of the Agulhas Current proper²². The average surface temperature measured by the buoy (Fig. 3*c*) was between 16 and 17°C until 26 December when the temperature rose rapidly to $>21^\circ\text{C}$. The period of this temperature rise coincided with the closest approach of the Sub-Tropical Convergence (Fig. 2) and the shift of the buoy circulation to a slightly more irregular path and slower circulation periods of 3.8 days (Fig. 3*b*).

By 8 January 1984 the surface expression of the eddy had become markedly elongated (Fig. 2) and by 11 January the buoy discontinued its gyrations, and drifted off in a direction parallel but adjacent to the observed route of the Agulhas Return Current^{25,26} (Fig. 2). The temperature measured by the buoy decreased from values $>21^\circ\text{C}$ to an average of 17°C . The eddy could not be identified in subsequent imagery of the sea surface.

It is probable that the first period of abrupt change in temperature and circulation characteristics of the buoy represents the inflow of warmer surface water from the Agulhas Return Current into the eddy across the Sub-Tropical Convergence. This heat-scavenging behaviour by an eddy has been demonstrated for Agulhas rings north of the Sub-Tropical Convergence²⁰ and for warm-core rings in collision with the Gulf Stream²⁷. In this case it may represent a potent mechanism for cross-frontal, meridional heat flux in the surface layers of areas

with high mesoscale turbulence in the Southern Ocean. The subsequent rapid decrease in temperature measured by the buoy may be indicative either of it having left the eddy or of the deterioration and fragmentation of the feature itself. The elongated surface expression of the eddy (Fig. 2) preceding the abrupt circulation change and the fact that temperatures measured by the buoy, although south of the Sub-Tropical Convergence ($<14^\circ\text{C}$), continued to be $>17^\circ\text{C}$ all favour the concept of bodies of water becoming detached from a no longer fully intact eddy.

An estimate of the total potential heat contribution by the eddy to its direct sub-Antarctic environment, with the T/S characteristics measured during the incursion into the feature (Fig. 1*b*), is $26.7 \times 10^{17} \text{ J}$. This is the total integrated heat content of the eddy over and above the heat content of a body with similar dimensions made up of sub-Antarctic water such as that between stations 124 and 125 (Fig. 1). It assumes that the eddy was more or less circular and takes into account only the water mass between 75 and 900 m, the maximum depth to which closely spaced temperature measurements were made. It is, therefore, an underestimate. The mean climatic mixed depth layer for September for the area is 75 m (ref. 28), and the mean ocean-to-atmosphere heat loss rate in the general area is high, 180 W m^{-2} (ref. 29). It is therefore assumed that the differential heat content of the upper 75 m would be preferentially lost to the atmosphere and has not been included in the estimate of cross-frontal heat exchange.

Long-term measurements of oceanic poleward heat transport have been suggested¹⁵ for Sub-Tropical Convergence areas such as the vicinity of the Agulhas retroflection and a set of such measurements that uses a network of deep-sea current-meter moorings and lasted two years, has just been completed. These measurements might successfully establish the production rate of warm and cold mesoscale eddies, but could conceivably overestimate the meridional heat flux if significant re-absorption of eddies occurred downstream of the measurement network, or could underestimate the heat flux if sporadic leaks of warm water to scavenging eddies, as reported here, took place. A reasonable understanding of the average natural history of such features is, therefore, required before a reliable estimate of their contribution to the heat flux can be made.

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Archaeon carbon reservoirs and their relevance to the fluid source for gold deposits

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It is commonly assumed^{1,2} that seawater-derived carbon in altered volcanic rocks is the only major pre-metamorphic carbon-in-carbonate reservoir in Archaeon greenstone belts. Thus carbonates from Archaeon gold deposits with stable isotope ratios too negative (median $\delta^{13}\text{C} \approx -3\%$) to have been derived from such carbon ($\delta^{13}\text{C}$ usually assumed to be near 0‰) have been interpreted as being derived from a local felsic magmatic source¹. Barley and Groves³, however, recognize two carbonate-alteration styles that predate regional metamorphism and gold mineralization in the Norseman-Wiluna greenstone belt of Western Australia: evidence is summarized below. These are seafloor alteration and fault-controlled regional alteration, which, as shown below, have completely different carbon isotope compositions. The latter (median $\delta^{13}\text{C} = -4.8\%$) implies a major juvenile carbon (CO_2) flux from the mantle during greenstone-belt evolution, and is thus of fundamental importance in itself. However, here we show why the mere existence of two carbon-in-carbonate reservoirs and variation in carbon isotope ratios of carbonates between individual gold deposits in one area, negate some of the fundamental assumptions used to support magmatic-fluid models¹.

Archaeon epigenetic gold deposits are a coherent group of structurally controlled, quartz-vein or disseminated deposits that have large down-dip or down-plunge continuity (>1 km), commonly without obvious vertical zonation^{4,5}. They have a characteristic metal association of $\text{Au} \pm \text{As} \pm \text{Ag} \pm \text{Sb} \pm \text{W} \pm \text{Te} \pm \text{B}$ with low to very low Cu, Pb and Zn contents⁶, and distinctive lateral zonation around hosting fractures or shear zones comprising (from the centre outwards) pyrite $\pm$ pyrrhotite $\pm$ arsenopyrite, K-mica $\pm$ Na-feldspar, and Ca-Fe $\pm$ Mg carbonate $\pm$ chlorite⁷⁻⁹. Most researchers agree that the deposits have a similar origin, and that Au was carried as reduced sulphur complexes in $\text{H}_2\text{O}-\text{CO}_2$, low salinity, near-neutral ore fluids¹⁰⁻¹² that were channelled by major fault/shear systems to dilational sites where Au

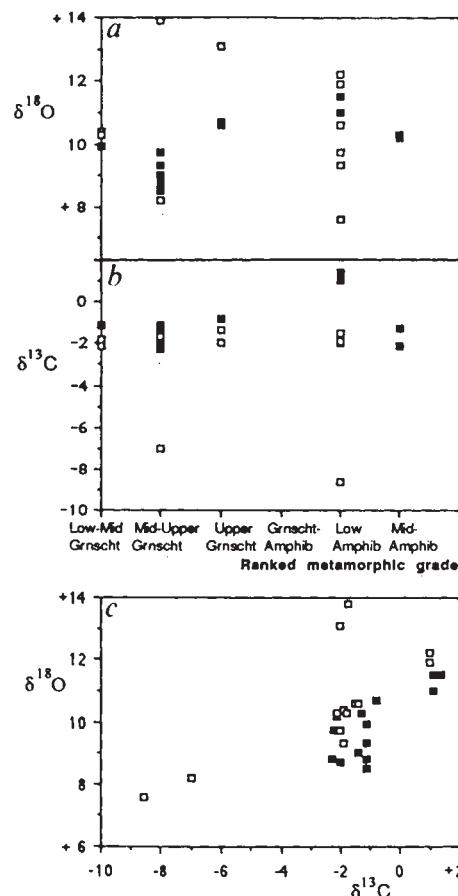


Fig. 1 $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ data for calcite in basalts representing sea-floor alteration (from Table 1 and ref. 16): a, $\delta^{13}\text{C}$ versus metamorphic grade; b, $\delta^{18}\text{O}$ versus metamorphic grade; c, $\delta^{18}\text{O}$ versus $\delta^{13}\text{C}$. Note: most carbonates, including all those with extreme values, come from surface samples and the scatter of data may, in part, reflect modern weathering despite extreme care in sampling. Interpillow (■); vesicle (□).

was deposited due to fluid/wallrock reactions^{4,5,13,14}. Despite this agreement, controversy continues concerning the source of ore components and fluid. This is because most fluid inclusion and stable isotope data cannot unequivocally discriminate between two end-member genetic hypotheses that stress either metamorphic^{4,6,10,15} or magmatic¹ derivation of ore fluids.

Three main arguments have been recently presented to support a magmatic origin for greenstone-hosted gold deposits. (1) Seawater-derived carbonate has $\delta^{13}\text{C}$ values that range around 0‰ and should yield carbonate with more positive $\delta^{13}\text{C}$ if that carbonate was derived from CO_2 liberated during metamorphic decarbonation; mean $\delta^{13}\text{C}$ values from carbonate alteration related to the two largest greenstone-hosted deposits (Hollinger-McIntyre, $-3.1 \pm 1.3\%$; Golden Mile, $-3.4 \pm 0.4\%$) thus appear too negative to have been derived from CO_2 metamorphically remobilized from seawater carbonate^{1,2}. (2) No alternative major carbonate reservoir (with a more suitably negative $\delta^{13}\text{C}$) has been identified^{1,2}. (3) The two largest gold deposits and the granodiorite-related Mink Lake Mo deposit have statistically indistinguishable $\delta^{13}\text{C}$ distributions, implying that all three are magmatic¹.

The argument that seawater carbonate was the only major carbon-in-carbonate reservoir (and hence the dominant contributor of CO_2 to metamorphic fluids) in greenstone belts², is not substantiated by our own database of 346 $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ determinations¹⁶. Analysis of variance and multiple comparisons (such as Scheffé's test) indicate that sea-floor alteration and

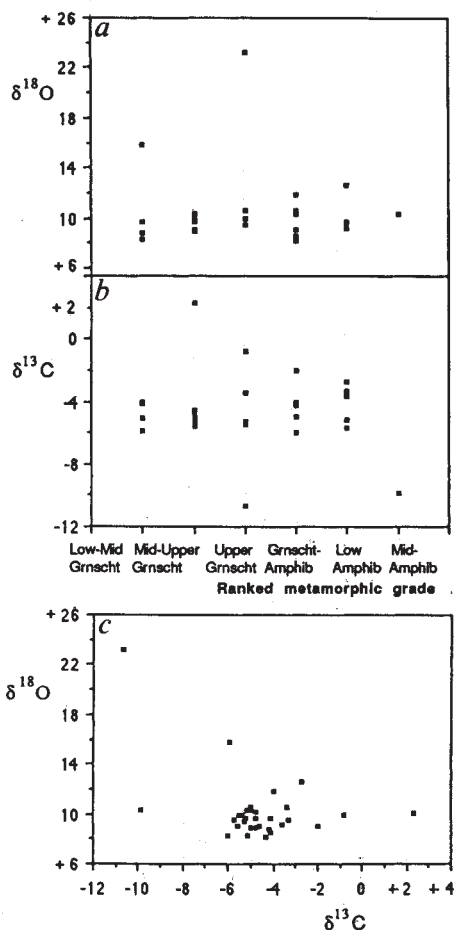


Fig. 2 $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ data for carbonates in komatiites affected by fault-controlled regional alteration: a, $\delta^{13}\text{C}$ versus metamorphic grade; b, $\delta^{18}\text{O}$ versus metamorphic grade; c, $\delta^{18}\text{O}$ versus $\delta^{13}\text{C}$. Note: as in Fig. 1, the scatter of data may, in part, relate to modern weathering although most samples are from drill cores.

fault-controlled regional alteration in the Norseman-Wiluna Belt of Western Australia³ have quite different $\delta^{13}\text{C}$ distributions¹⁶. Two carbonate reservoirs are also implied by $\delta^{13}\text{C}$ data for the Murchison greenstone belt, South Africa¹⁷, whereas $\delta^{13}\text{C}$ values of -1.5 to $+0.3\%$ from Hollinger¹⁸ (considered "atypical" by Burrows *et al.*¹) hint at more than one carbon reservoir in that mineralized environment also.

Sea-floor alteration (including carbonation), new data for which are presented in Table 1, was controlled by the poros-

Table 1 New carbon and oxygen isotope data for calcites filling vesicles and in interpillow positions in basalts remote from gold mineralization, interpreted to represent seafloor alteration

Number	Location	Metamorphic grade	Type	$\delta^{13}\text{C}^*$	$\delta^{18}\text{O}^*$
102470	Lake Cowan	Low amphibolite	Large vesicle	-2.0	9.7
102472	"	"	"	-1.9	9.3
102473	"	"	"	-1.5	10.6
102474	"	"	Interpillow	1.4	11.5
102475	"	"	"	1.1	11.0
102476	"	"	Large vesicle	1.0	12.2
102477	"	"	"	1.0	11.9
102478	Marshall Pool	Low-mid greenschist	"	-2.1	10.3
102479	"	"	"	-1.9	10.4
102480	"	"	"	-1.8	10.3
102481	"	"	Interpillow	-1.1	9.9

Additional data for similar carbonates, analytical methods and uncertainties in ref. 16.

Carbon and oxygen isotope values are reported in per mil (‰) relative to the PDB ($\delta^{13}\text{C}$) and SMOW ($\delta^{18}\text{O}$) standards, respectively.

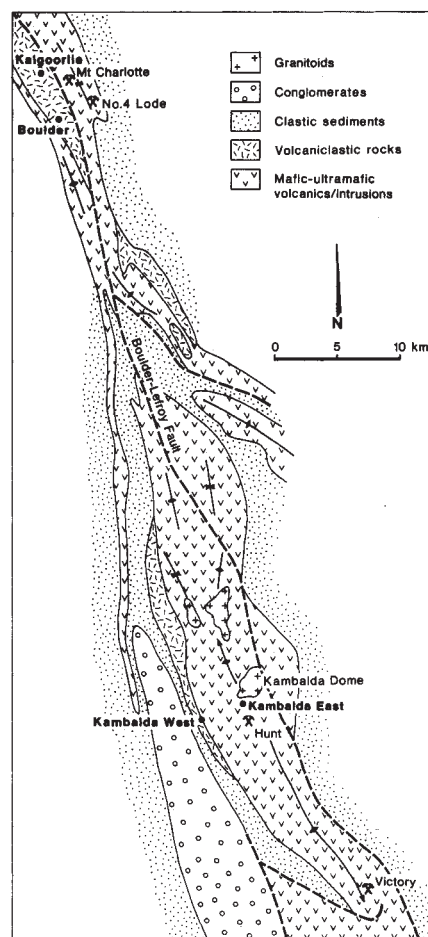


Fig. 3 Geological sketch map of the Kalgoorlie-Kambalda district showing the Boulder-Lefroy Fault and the location of gold deposits for which $\delta^{13}\text{C}$ data are given in Table 3. Adapted from Muhling³⁸.

ity/permeability of volcanic units and their stratigraphic position and is clearly a synvolcanic phenomenon³. In this process: (1) most carbonate is interpillow or vesicle calcite in the host basalts; (2) the most intense alteration occurs over restricted vertical profiles towards the top of the volcanic succession; (3) carbonation at lower stratigraphic levels is limited; and (4) overall addition of carbon from sea water to the greenstone pile is small (probably $<0.1\%$) (ref. 19). The spread of $\delta^{13}\text{C}$ values around zero¹⁶ (Table 1) is consistent with a sea-floor alteration origin²⁰⁻²². Although the samples in Table 1 cover only a limited range of metamorphic grade, coupled ^{13}C and ^{18}O depletion indicative of partial metamorphic decarbonation^{23,24} is lacking (Fig. 1); any CO_2 contribution to metamorphic fluids is therefore most likely to have been via dissolution reactions or, less likely, by complete decarbonation. High-temperature dissolution in low-carbonate rocks will produce CO_2 that is isotopically similar to original carbonate²⁵; thus sea-floor carbonate (unless with a more negative $\delta^{13}\text{C}$ than normally accepted) cannot represent the major source of carbon in gold-related carbonates at Hollinger and the Golden Mile ($\delta^{13}\text{C} \approx -3\%$)¹.

Fault-controlled regional alteration contrasts with sea-floor alteration in that: (1) carbonation is far more intensive, in zones round large-scale faults; (2) it mainly affects ultramafic sequences low in the stratigraphic succession, although porphyry intrusions and basalts are locally affected; (3) dolomite and magnesite are more common than calcite; and (4) carbonated rocks can have high ($>10\%$) CO_2 contents.

The fault-controlled regional alteration is far more widespread

Table 2 Summary statistics for carbon isotope compositions of carbonate minerals from sea-floor alteration and fault-controlled regional alteration in the Norseman-Wiluna Belt and from gold-related carbonate alteration in gold deposits in the Kalgoorlie and Kambalda gold camps

Description	Range	n	$\delta^{13}\text{C}\%$ Mean $\pm$ st.dev.*	Median	$\sqrt{b_1}$ Skew†	$[b_2 - 3]$ Kurtosis†
Sea-floor alteration of basalts						
Interpillow calcite	-2.3 to 1.4	15	-1.0 $\pm$ 1.2	-1.1	+0.97	-0.35
Vesicle calcite	-8.6 to 1.0	13	-2.3 $\pm$ 2.7	-1.9	-1.24	+1.03
—excluding outliers‡	-2.0 to -1.4	9	-1.8 $\pm$ 0.2	-1.9	+0.62	-0.87
Fault-controlled regional carbonation						
Carbonated komatiites	-10.7 to 2.3	32	-4.6 $\pm$ 2.2	-4.8	+0.13	+3.33
—excluding outliers‡	-6.0 to -2.0	28	-4.6 $\pm$ 1.0	-4.8	+0.89	+0.35
Gold-related alteration in gold camps						
Hunt mine—porphyries	-6.6 to -4.7	8	-5.1 $\pm$ 0.6	-5.1	-1.40	+0.94
Hunt mine—Footwall Basalt	-6.4 to -4.4	3	-5.3 $\pm$ 0.9	-4.7	-0.64	-1.50
Victory/Defiance—porphyries	-8.7 to -4.6	22	-7.1 $\pm$ 1.3	-7.3	+0.43	-0.98
Mt Charlotte—Reward	-7.6 to 12.7	21	-4.8 $\pm$ 4.4	-6.0	+3.2	+10.6
—excluding outliers‡	-7.6 to -1.6	20	-5.7 $\pm$ 1.8	-6.1	+1.16	+0.45
Mt Charlotte—Charlotte	-7.9 to 8.8	39	-5.0 $\pm$ 3.2	-6.0	+2.81	+8.63
—excluding outliers‡	-7.9 to -2.8	36	-5.9 $\pm$ 1.3	-6.1	+0.77	-0.13
golden Mile—No. 4 lode	-4.1 to -1.5	21	-3.4 $\pm$ 0.4	-3.5	+1.58	+2.52

* Primary data in ref. 16 and Table 1. Values only given for comparison with the summary statistics of Burrows *et al.*¹ Means and standard deviations are not reliable location and scale estimates for such non-normally distributed data, which also include gross outliers. (Having demonstrated a similar degree of non-normality for their own data, Burrows *et al.*¹ should also have used medians as location estimates.)³⁹

† Both parameters are zero for a normal distribution. Values indicating non-normality at the 95% and 99% levels are respectively **emboldened** and **emboldened and italicized**. Note therefore that each data set is significantly skewed, leptokurtic, or both. Non-parametric normality tests (for example, χ^2 and the Kolmogorov one-sample test) indicate the same conclusion.

‡ Recognized by Grubbs' maximum studentized residuals and Dixon's criteria³⁹.

than even the most extensive gold-related carbonation at Kalgoorlie²⁶, and some major zones of regional carbonation contain no significant gold deposits. Available constraints on timing indicate that the regional alteration predated both the peak of regional metamorphism and gold mineralization. At Agnew, for example, mineralogical variations in metamorphosed dunites relate to infiltration by CO_2 -rich fluids generated by decarbonation (or dissolution) of pre-metamorphic talc-carbonate rocks²⁷. Similarly, in komatiites at Redross near Widgiemooltha²⁸, and at Windarra, carbonate porphyroblasts are wrapped by or rotated in the metamorphic foliation, and later amphibole overgrowths occur on talc-carbonate schists. This contrasts with both lode-scale⁸ and district-scale²⁶ gold-related carbonation which commonly involve at least partial replacement of metamorphic assemblages.

The fault-controlled carbonate reservoir is more likely than a sea-floor reservoir to have provided CO_2 for metamorphic fluids involved in gold mineralization, because it occurs at a more suitable (lower) stratigraphic level, is concentrated along crustal structures that subsequently controlled the distribution of gold deposits (for example, the Boulder-Lefroy Fault), and can explain the anomalously CO_2 -rich auriferous fluids²⁹. The narrow $\delta^{13}\text{C}$ range of carbonated komatiites (-6 to -2‰) and the median value of -4.8‰ (Table 2) suggests that the fault-controlled alteration involved CO_2 of mantle or magmatic origin^{25,30}, channelled by transcrustal fault systems³¹. This is also consistent with its regional extent, close relationship to major fault zones, and gross independence of specific igneous intrusions that are exposed in the terrane. Figure 2 (like Fig. 1) reveals no systematic coupled ^{13}C and ^{18}O depletion with increasing metamorphic grade, and therefore any carbon contribution from fault-controlled carbonation to auriferous ore fluids again is most likely to have been via equilibrium dissolution rather than partial decarbonation. Thus it will be difficult to distinguish between models suggesting reworking of mantle-derived carbonate into metamorphic fluids and direct involvement of mantle fluids. The former is, however, favoured by the much greater variation between $\delta^{13}\text{C}$ values of carbonates from

gold deposits in similar host rocks within a single district (see also deposits in the Abitibi Belt³²) than for carbonates from regional alteration zones along length (>400 km) of the Norseman-Wiluna Belt (Table 2)¹⁶. The provinciality of lead isotope data³³ also implies the variable involvement of metamorphic fluid from old granitic basement to the greenstone belts. Thus, fault-related carbonation in the Norseman-Wiluna Belt constitutes a spatially and stratigraphically suitable reservoir of carbon with prerequisite $\delta^{13}\text{C}$ ($\approx -5\%$) to yield gold-related carbonate ($-5 \pm 2\%$) by metamorphic reworking.

The final argument for the magmatic model¹ assumes that two sets of carbonates with statistically indistinguishable $\delta^{13}\text{C}$ distributions must have the same origin. This can be challenged statistically³⁴, but, more importantly, it fails to allow for geological convergence: many rocks of different origins are mineralogically and/or chemically similar (for example ortho- and para-amphibolites), and isotopic convergence can occur in, for example, $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, where 'high' values can reflect either crustal involvement (as in S-type granites³⁵) or an ancient metasomatized mantle source (as in lamproites³⁶). We therefore suggest that although statistically different isotopic distributions (as in Table 2) must to some degree imply different sources or depositional environments, the converse does not apply: statistically similar distributions are only a necessary, and not a sufficient, criterion for precisely equivalent origins.

The argument based on statistical similarity is also invalid because carbonates from deposits associated with the same major controlling structures (or even from the same gold camp)⁶ can have different $\delta^{13}\text{C}$. For example, the Kalgoorlie and Kambalda gold camps are both spatially and probably genetically^{8,37} related to the Boulder-Lefroy fault zone (Fig. 3), but median $\delta^{13}\text{C}$ for alteration-related carbonates at Hunt and Victory (both in the Kambalda camp) and for No. 4 Lode, Golden Mile (in the Kalgoorlie camp), all contrast with medians for the Charlotte and Reward orebodies at Mt Charlotte (also in the Kalgoorlie camp; Table 2). These differences between geologically related deposits may reflect variation in the proportions of oxidized and reduced carbon species in solution and/or variable mixing

of CO₂ from dissolution of the two carbon-in-carbonate reservoirs in space or time. Whatever their origin, the mere existence of differences in $\delta^{13}\text{C}$ between related deposits precludes genetic conclusions from statistical comparison of geologically unrelated deposits.

In summary, our data point to two major pre-mineralization carbon-in-carbonate reservoirs in the Norseman-Wiluna Belt: the one other well-studied greenstone terrane in South Africa provides similar results¹⁷. One of these was probably developed by mantle degassing and consequent carbonation along the same major fault zones that subsequently controlled the location of epigenetic gold deposits. Metamorphic reworking of this carbon-

ate can explain: (1) the high CO₂ content of auriferous fluids, and (2) local variations and negative $\delta^{13}\text{C}$ values in gold-related carbonates. Local magmatic sources of carbon are not a necessary requirement, although an earlier, ultimately magmatic, mantle source for at least some of the carbon remains highly likely.

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Host detection by chemically mediated associative learning in a parasitic wasp

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Parasitic insects use chemical cues to locate their hosts, and prior experiences can modify their responses to these odours¹⁻⁴. Females of the parasitic wasp *Microplitis croceipes* experienced by contact with host faeces, orient and fly upwind to odours from their hosts, larvae of the moth *Heliothis zea*⁵. We use flight tunnel studies to show that associative learning occurs during encounters with host faeces. When females touch the faeces with their antennae they learn to recognize and subsequently fly to various volatile odours, even novel and otherwise unattractive odours like vanilla, associated with the faeces. They link these volatile odours with a water extractable nonvolatile chemical in the faeces, evidently a host-specific recognition cue. The association of tracking cues with host by-products, without the need for direct contact with the host, is a valuable adaptation for locating cryptic and evasive hosts.

When females of *M. croceipes* are presented with fresh faeces or the water extract of faeces (Fig. 1) on filter paper, they immediately rub the material with their antennae and sometimes probe it with their abdomens. This intense examination continues for several minutes unless interrupted. Subsequently,

these females exhibit characteristic host-seeking flight responses to attractants in a wind tunnel. Females presented with the hexane extract (Fig. 1) do not exhibit the sustained antennal rubbing and subsequently do not respond to attractants any better than naive females (Fig. 2). When the hexane and water extracts were tested as attractants in the wind tunnel, the hexane extract was significantly better than other sources including fresh faeces (Fig. 3).

As the water extract showed some attraction in the wind tunnel, we tested whether actual contact with the material is a necessary part of the experience, or whether exposure to certain volatiles simply increases the probability that females will recognize and subsequently respond to those volatiles. Females were exposed for 1 min to volatiles from the water extract on filter paper in closed Petri dishes, but were prevented from contacting the filter paper by a piece of cheese cloth. Of 20 females exposed in this way, none responded to the hexane extract in the wind tunnel, whereas 80% of a similar group of females allowed to contact the water extract on filter paper with only their antennae did respond. Thus, water extractable, non-volatile components of host faeces, contacted directly by *M. croceipes* female antennae, provide a cue causing the females to respond subsequently to volatile attractants.

The most likely explanation of the flight response of the parasitoid to the hexane extract subsequent to antennal rubbing of the water extract was considered to be either one or a combination of sensitization, associative learning and/or priming. As prior exposure to hexane extract did not improve response and actual contact with the water extract was required (indicating a non-volatile material), sensitization seemed an unlikely explanation. We adopted associative learning as our working hypothesis on the assumption that the limited attraction of the water extract (Fig. 3) was a result of contamination with hexane soluble volatiles. Thus, contact with the water extract (or raw faeces) would result in the association of volatile hexane soluble

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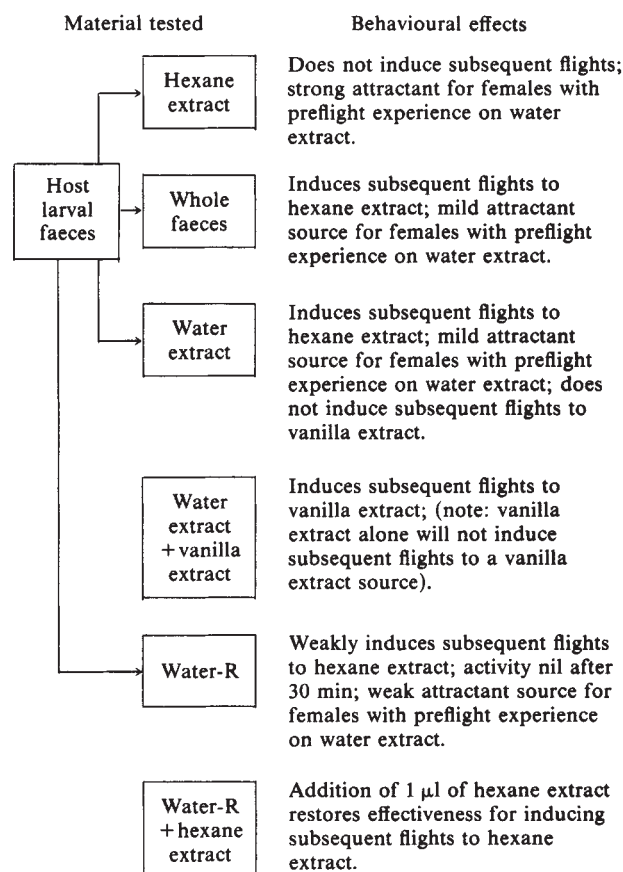


Fig. 1 Summary of the effects of *H. zea* larval faeces and extracts of the faeces on the behaviour of *M. croceipes* females.

Methods. Faeces were collected from third instar larvae of *H. zea* fed on cowpea leaves. The water extract was obtained by soaking 1 g of fresh faeces in each of three aliquots of high pressure liquid chromatography (HPLC) grade water (J. T. Baker) (20 ml total) for 3 h with frequent vigorous shaking. Then the residual faeces was extracted in the same way with a total of 10 ml of hexane to provide the hexane extract. To obtain the water-R extract, 1 g of fresh faeces was extracted with pentane in a soxhlet extractor for about 300 h. Then the residual faeces was extracted with three aliquots of HPLC water (20 ml total) as before.

chemicals with a nonvolatile water soluble host-recognition material. This was supported by the fact that exposure to the water extract (water-R) of the residue from faeces exhaustively extracted with pentane resulted in very weak responses, whereas activity could be restored by the addition of hexane extract (Fig. 1).

To test this associative learning hypothesis, we added a novel odour, vanilla extract (Nielsen-Massey) to filter paper together with the water extract. A significant number of females exposed to this combination subsequently were attracted to vanilla odour in a flight tunnel. Naive females or females exposed to the water extract without vanilla did not fly to the vanilla source (Fig. 4). This shows that associative learning does occur. When exposed to vanilla extract alone, females do not rub it with their antennae, and subsequently are not attracted to a vanilla source.

Taken together with the fact that the hexane extract alone does not condition flight responses, these results show that a nonvolatile water extractable component (unconditioned stimulus) acts together with hexane soluble volatile chemicals (conditioned stimuli) to elicit antennal rubbing and the association process. The parasitoid subsequently flies to sources of the volatiles thereby learned. An analogous process has been

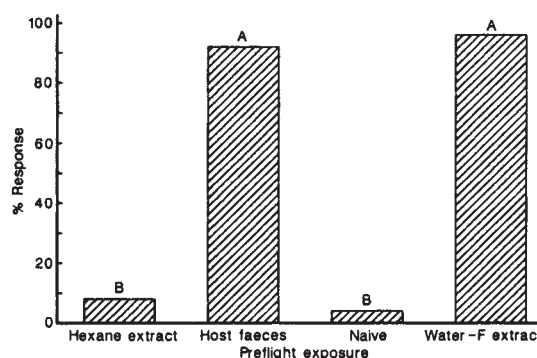


Fig. 2 Flight response of *M. croceipes* females to hexane extract of *H. zea* larval faeces after no experience (naive) or contact with fresh host faeces, with water extract of host faeces (water-F), or with hexane extract of host faeces. Bars capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K-ratio t -test, minimum significant difference = 18.9%, $n = 96$ wasps (four replications, four treatments, six wasps per replication per treatment). **Methods.** *M. croceipes* were reared on the larvae of *H. zea* fed on an artificial diet¹³. Females were allowed to mate and then held in $30 \times 30 \times 20$ cm cages until bioassay. The wind tunnel and general bioassay procedure have been described previously⁵. All flight responses were tested at $26-28^\circ\text{C}$, at a wind speed of 56 cm s^{-1} and at a light intensity of $2,500\text{ lx}$. The hexane extract ($14\text{ }\mu\text{l} = 1.4\text{ mg}$ of faeces) used as the attractant source was pipetted onto a triangular ($9.5 \times 6.5\text{ cm}$) piece of Whatman number 1 filter paper suspended at the centre of the upwind end of the tunnel. Females, tested individually, were released from a 4-dram shell vial 80 cm downwind and in the plume from the odour source. The behaviour of the females in the wind tunnel was observed until a complete flight occurred or five minutes elapsed. A complete response involved the host-seeking flight behaviour described by Drost *et al.*⁵ and included approach and landing on the odour source. For each observation, a female was given two chances to make a successful flight. Materials to which females were exposed before flight testing— $28\text{ }\mu\text{l}$ ($= 1.4\text{ mg}$ faeces) of water-F extract, $14\text{ }\mu\text{l}$ hexane extract, or $\sim 2\text{ mg}$ of host faeces—were placed on a 12.5 cm diameter sheet of Whatman number 40 filter paper in a Petri dish. Females were allowed to walk directly from a vial onto the test material and then to investigate the material with their antennae for 45 s.

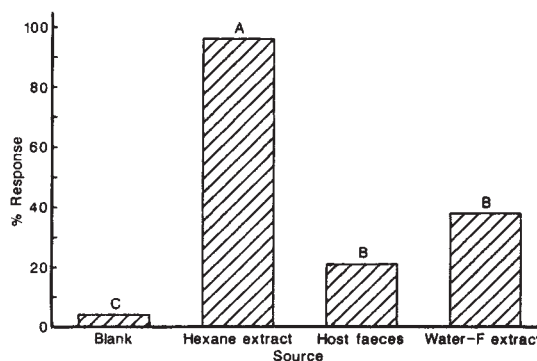


Fig. 3 Flight response of *M. croceipes* females to pure hexane (blank), fresh host faeces, water extract of host faeces (water-F), and hexane extract of host faeces, after contact with water extract of host faeces. Bars capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K-Ratio t -test, minimum significant difference = 17.2%, $n = 96$ wasps (four replications, four treatments, six wasps per replication per treatment). **Methods.** See legend to Fig. 2.

shown in rats, where novel odours were potentiated when the taste receptors were contacted⁶. To show that the water extractable substance (a kairomone⁷) does not occur in the larvae of *Trichophisia ni*, a non-host species, females were exposed, both with and without the presence of hexane extracts of *H. zea* (host) faeces, to water extracts of faeces of the larvae of *T. ni* fed on cowpea leaves. These females did not respond to hexane

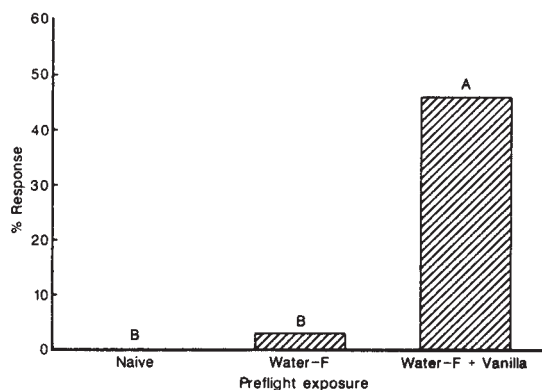


Fig. 4 Flight response of *M. croceipes* females to a source of vanilla extract (Nielsen Massey) in a wind tunnel after no experience (naive), or contact with water-F, or water-F plus vanilla extract. Bars capped by different letters are significantly different, $P < 0.01$, Waller-Duncan K -Ratio t -test, minimum significant difference = 12.4%, $n = 90$ wasps (three replications, three treatments, ten wasps per replication per treatment).

Methods. This experiment was conducted in the same way as previous experiments (Figs 2 and 3). Pure vanilla extract (80 μ l) was pipetted onto a piece of Whatman number 1 filter paper and used as the attractant source as described before. Materials used for preflight conditioning of the females were prepared as before. When water-F and vanilla were combined, 28 μ l of water-F was first placed on the filter paper and then 1 μ l of vanilla extract was added to the centre of the moist spot.

extracts of either *H. zea* or *T. ni* faeces.

Our findings show that the host-seeking behaviour of *M. croceipes* females is mediated by both a nonvolatile, contact chemical and by volatile chemical cues. The nonvolatile substance is water extractable and distinct from the hexane soluble volatile cues that attract females. The nonvolatile compound is also host specific. The volatile attractants may be plant-derived. The role of the relatively nonvolatile, hexane soluble kairomone, 13-methylhentriacontane, previously identified for this parasitoid⁸, is not known. It may facilitate the association process described here by enhancing the antennal rubbing behaviour.

M. croceipes parasitizes *Heliothis* species, which are highly polyphagous. Thus the ability to locate its hosts among other plant feeders in diverse systems of vegetation is crucial. Learned responses to varying trail odours through association with a host recognition kairomone in the host faeces may give *M. croceipes* females an adaptive advantage. Associative learning in connection with host-detection by parasitoid and plant feeding insects has been shown by a number of authors^{2-5,9-12}. Contact with the target organism (or a model) and subsequent oviposition, however, generally was necessary. We believe this to be the first demonstration of associative learning in insects where encounter with the target organism is not involved. This phenomenon provides a valuable way of finding hosts or prey species and may be widespread in parasitic and predatory systems as the target organisms (but not their by-products) often elude the searching parasitoid or predator.

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Perception of three-dimensional structure from motion in monkey and man

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Information on motion is important for the determination of the three-dimensional (3-D) structure of the environment for both human and non-human primates¹⁻⁸. For example, if a person were to close one eye and look at an evenly illuminated, irregularly shaped object, he would be unlikely to guess its shape correctly. But if the object is moved about, the correct shape immediately becomes apparent⁹. Little is known about how the primate visual system actually does this, although various theories have been proposed^{7,8,10-15}. We have developed novel, highly controlled motion stimuli to use with psychophysical and physiological techniques to study how 3-D structure is obtained from motion. We show that the Rhesus monkey can detect 3-D structure from motion in the same way as human subjects. Furthermore, the dependence of both species on certain parameters of the display shows that information is integrated both spatially and temporally for this higher visual function.

We have defined the ability to extract structure from motion in terms of a reaction-time task. The subject is told, or conditioned, to pull on a lever at the onset of the stimulus and to try to release it within 1 s after the stimulus changes at a randomly selected time¹⁶. In these experiments, the stimulus changes from a control with no structure to a test with 3-D structure.

The control unstructured and the test 3-D structured stimuli are computed from the parallel projection of points covering the surface of a cylinder (Fig. 1). For the structured stimulus the cylinder revolves at a fixed angular velocity of 35 degrees s^{-1} . The horizontal velocity of the projected points varies as a sinusoidal function of their horizontal location on the screen. Points at the centre move fastest whereas those at the edges move slowest. The unstructured stimulus is generated by computing the velocity trajectories of the points as described above and displacing each trajectory using a randomly selected distance chosen from a uniform distribution equal to the width of the display. This allows conservation of the velocity distribution while destroying the spatial relationship of the points with respect to each other.

Each point is displayed for a pre-selected duration defined as its lifetime^{7,8,17}. At the end of the point's lifetime it disappears and randomly reappears at a new location on the screen to begin a new motion trajectory. The beginning of the lifetimes are staggered so that the points asynchronously flicker on and off. This effect can be imagined by visualizing the reflections of sunlight on a waterfall. Any possible form cues are constantly changing. The point density across the screen is also kept constant leaving only the change in the structure of the display as a cue for the release of the key. The change from unstructured motion to structured motion for each point occurs during the relocation of that point. The total time to switch from the unstructured to the structured display is equal to the point lifetime.

The three human and three monkey subjects tested were able to detect the change from completely unstructured motion to a completely structured motion at the 90-100% correct level (the number of points was 128; point lifetime was 532 ms). To quantify this, psychometric functions were obtained by plotting the

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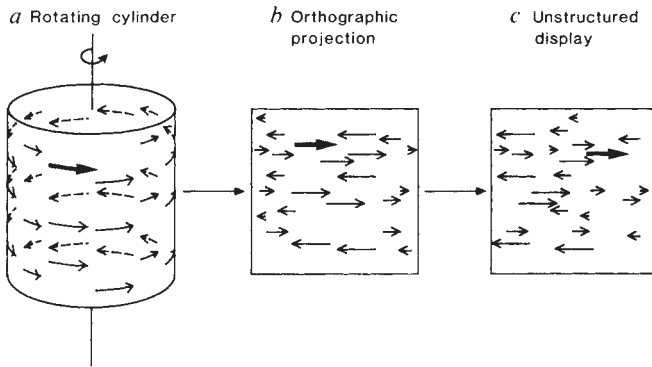


Fig. 1 Generation of the motion displays. The velocity of each point is indicated by the length of the arrows. *a*, The stimuli are generated by first computing the location of points on the surface of the rotating cylinder for each instant in time. *b*, The points are then parallel (orthographically) projected onto a plane perpendicular to the observer's line of sight. This is the 'structured display'. *c*, The 'unstructured display' is computed by taking each point's motion trajectories from the test display and displacing them randomly in a window equal to the width of the display. The fate of an individual motion trajectory for a point crossing the front of the display is shown by the bold arrow. The point density on the surface of the display is kept constant; each point is displayed for a finite amount of time. At the end of this time, the point vanishes and reappears randomly on the screen to follow a new motion trajectory.

percentage of trials where the change was detected within the reaction-time window as a function of the structure of the test display (Fig. 2a). The structure of the test display was reduced by increasing the range over which the points were randomly displaced. (The fraction structure of the display is defined as one minus the ratio of the range over which the trajectory displacement can occur divided by the width of the display.) Both species have similar psychometric functions for the dependence on the fraction of structure in the display (Fig. 2a). These experiments therefore provide evidence that non-human primates can detect the change from unstructured to structured motion and that this ability is similar to that of humans.

A control experiment was performed to ensure that the subjects were not using local analysis of the motion-flow field to perform this task. When the display was masked so that only the central 1/25th of its area was visible, the three human subjects and two monkey subjects tested were unable to detect the change from no structure to complete three-dimensional structure ($P < 0.01$, Kolmogorov-Smirnov test for each of the five subjects). This result shows that local artifactual cues such as a change in the distributions of speeds were insufficient to perform the task. The subjects were probably not able to detect structure from motion in the smaller areas of the surface because too few points were visible to perform the computation.

Computational and psychophysical studies have suggested that the point lifetime^{7,8} and number of points^{5-8,14,15} in the display are important constraints for abstracting structure from motion. It was found that the subjects' performance of the task declined for shorter point lifetimes (Fig. 2b). When 128 points were viewed and the lifetime was shorter than 75 ms and 100 ms for monkeys and humans respectively, they were unable to do the 3-D task. It was found that both species' performance was reduced by ~40–70% when less than 32 points were viewed.

Our results show in two primate species that more than four times the theoretical minimum number of points (based on geometric considerations of the structure-from-motion theorem¹⁴) were necessary to abstract structure from motion in the primate visual system with the flickering dot display. The observation that the minimum point life could be as short as

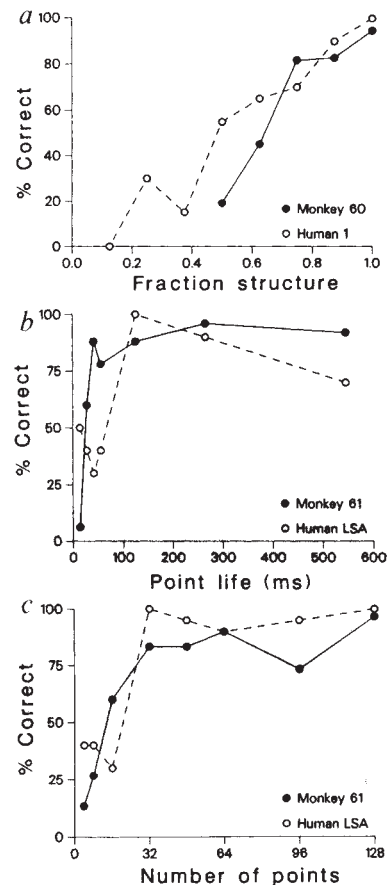


Fig. 2 A human subject's and Rhesus monkey's performance as a function of the parameters of the display. *a*, The percentage of trials where the subject released the key within the requisite time window is plotted as a function of the fraction of structure. The two primates have similar psychometric functions that have a decrease in detection when the fraction of structure is less than ~0.65. There was no difference between the human and monkey subjects (χ^2 for independent samples, $P < 0.05$). In this experiment, 128 points were viewed with a point lifetime of 532 ms; the cylinder revolved at 35 degrees s^{-1} ; the display was refreshed at 35 Hz. The ability of the monkey and human subjects to perform the task depended on both the point lifetime (*b*) and number of points (*c*) of the display. The significant difference in minimal point lifetime required to successfully perform the task between the human and primate subject is probably due to the additional training given the monkey subjects. It can be seen that decreasing the number of points makes it more difficult for the subjects to perform the task. In *b* and *c*, the fraction of structure was 0.875; the refresh rate was 70 Hz; the cylinder revolved at 35 degrees s^{-1} .

100 ms has important implications for selecting algorithms to model the structure-from-motion process. The average reaction time for detecting the shift from no structure to structure was 600–1000 msec depending on the individual. Thus algorithms¹⁵ that require the positions of points continuously during the entire computation period would fail under these conditions. Rather, in our experiments, representations of entire surfaces appear to be computed from the flow field; points can exist momentarily anywhere on the surface and still be used for the computation.

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Brain-derived neurotrophic factor prevents neuronal death *in vivo*

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Developing vertebrate neurons are thought to depend for their survival on specific neurotrophic proteins present in their target fields¹. The limited availability of these proteins does not allow the survival of all neurons initially innervating a target, resulting in the widely observed phenomenon of naturally occurring neuronal death¹. Although a variety of proteins have been reported to promote the survival of neurons in tissue culture²⁻⁴, the demonstration that these proteins increase neuronal numbers and/or decrease neuronal death *in vivo* has only been possible with nerve growth factor (NGF)⁵⁻⁷. The generalization of the concept that neurotrophic proteins regulate neuronal survival during normal development critically depends on the demonstration that the survival of neurons *in vivo* can be increased by the administration of a neurotrophic protein different from NGF. We report here that this is the case with brain-derived neurotrophic factor, a protein of extremely low abundance purified from the central nervous system.

Brain-derived neurotrophic factor (BDNF) is a small, basic protein⁸ that supports the survival of various neurons *in vitro*, in particular of primary sensory neurons originating from the neural crest and ectodermal placodes⁹. Given the limited availability of BDNF, we decided to inject it into quail embryos: not only is the size of these embryos small when compared, for example, with those of the chick, but also their development is more rapid (16 versus 21 days¹⁰). We first counted the number of neurons at various embryonic stages in both the nodose ganglion of placodal origin¹¹ and a dorsal root ganglion of neural crest origin¹¹. It was found that in both ganglia, many neurons are lost during development (Fig. 1): about 30% and 38% for the nodose and dorsal root ganglia respectively. Either NGF (10 µg) or BDNF (1 µg) were injected daily from embryonic day 3 until day 7. Embryos were killed at day 8 and the neurons counted (Table 1). Upon dissection, the only macroscopically visible effect was an increase in the size of the sym-

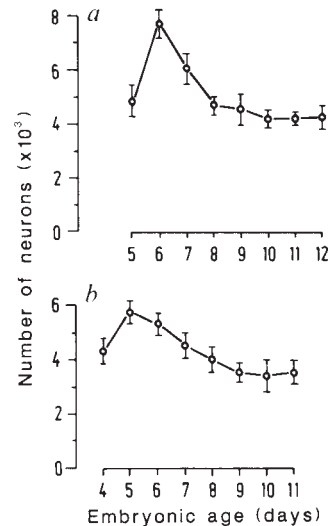


Fig. 1 Time course of naturally occurring cell death in *a*, quail dorsal root ganglion (DRG, 25th according to ref. 18) and *b*, nodose ganglion (NG). The values are means $\pm$ s.d. of 4-8 ganglia. No left and right differences were found for either ganglia.

Methods. Quail embryos were staged according to Zacchei¹⁰, the ganglia fixed in 4% formaldehyde, embedded in paraffin, sectioned at 6 µm and stained with thionine. All neurons (clearly identified as such by their large size and intensive staining) containing at least one nucleolus were counted in alternative sections at a magnification of $\times 250$. The number of neurons obtained was multiplied by 2 and no corrections were made: only 4% of the nuclei were found to contain more than one nucleolus and the size of one nucleolus (1-2 µm) is substantially smaller than the section thickness⁷. We directly checked that split nucleoli did not affect neuronal counts by comparing ganglia sectioned at either 6 or 10 µm: no significant differences in neuronal numbers were found²⁰.

pathetic chain in the NGF-treated embryos. No such effects were seen with BDNF. As expected from previous studies with the chick⁶, it was found that repeated injections of NGF rescued many neurons in the dorsal root ganglion: the number of neurons was significantly higher at day 8 than in control, and corresponded roughly to the peak value found before the period of neuronal death. Similar results were obtained with BDNF, supporting an earlier suggestion, based on findings¹² *in vitro*, that some primary sensory neurons, corresponding in this case to the population normally eliminated, could survive either with an excess of (peripherally derived) NGF or (centrally derived) BDNF. To establish directly that exogenous BDNF prevents normally occurring cell death *in vivo* (a fact firmly established *in vitro*^{8,9,12}), we also counted degenerating neurons (Table 2). We found that the total number of degenerating neurons could be significantly reduced by administering BDNF. In the nodose ganglion, as in the dorsal root ganglion, injections of BDNF resulted in an increased number of neurons at day 8 when compared with controls. Again, this number corresponds to the highest number of neurons found before cell death. No effects of NGF were seen on the nodose ganglion.

From the results that increased neuronal numbers are found in dorsal root and nodose ganglia after application of exogenous BDNF, we conclude that this protein, like NGF in peripheral target fields¹³, is normally present in limiting amounts in the central target fields of the primary sensory neurons. It is known from studies in the chick that the presence of the central target, the neural tube, is essential for the development of the dorsal root ganglion: removal of the neural tube¹⁴ or interposition of a membrane between the neural tube and the dorsal-root-ganglion anlage¹⁵ both result in the death of neural-crest-derived

Table 1 Effects of NGF and BDNF on neuronal numbers in dorsal root ganglion (DRG) and nodose ganglion (NG)

	DRG	NG
Control (E8)	4,767 ± 294 (n = 7)	4,016 ± 407 (n = 8)
BDNF (E8)	7,260 ± 449 (n = 5)*	5,711 ± 433 (n = 6)*
NGF (E8)	6,773 ± 705 (n = 11)*	3,969 ± 390 (n = 12)
Maximal neuronal numbers	7,574 ± 545 (n = 6, E6)	5,813 ± 444 (n = 8, E5)

BDNF (1 µg) or NGF (10 µg) were applied once daily onto the chorioallantoic membrane in 50 µl phosphate-buffered saline containing 1 mg ml⁻¹ horse-heart cytochrome C. Controls were treated with the same solution without factor. NGF was purified as described, using a 55% saturation ammonium-sulphate precipitation²¹. BDNF was purified as published⁸, with the following modifications: pig brains (in 1 kg portions) were homogenized in 2.5 l 0.2 M sodium-phosphate buffer, pH 6.0 containing 1 mM EDTA, 2 mM PMSF (phenylmethanesulphonyl fluoride) and 0.1% Triton X-100. After 40 min centrifugation at 8,000 r.p.m. in a Sorvall GS-3 rotor, the supernatant (diluted 1 to 1 with water, pH adjusted to 6.0) was stirred for 90 min with carboxymethyl cellulose (200 ml). The washed cellulose was poured into a column, run overnight with 0.1 M sodium phosphate pH 6.0 containing 0.13 M NaCl, and eluted as described⁸. The eluate (corresponding to 10 kg starting material) was dialysed, applied to and eluted from a 120 ml hydroxylapatite column as described⁸. The active fractions from two such columns (20 kg starting material) were pooled, made 1.0 M by adding solid potassium phosphate (final pH 6.8) and applied to two columns in series: 40 ml Octylsepharose followed by 6 ml Phenylsepharose (flow rate: 40 ml h⁻¹). After washing with 200 ml potassium-phosphate buffer, the Octylsepharose column was disconnected and the Phenylsepharose column eluted with 11 ml 0.1 M potassium-phosphate buffer pH 6.8. After concentration to 1.6 ml by evaporation, this eluate was applied at 0.1 ml min⁻¹ onto a C8 microbore reversed-phased column (Aquapore, Applied Biosystems) equilibrated with 0.1% trifluoroacetic acid. Proteins were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (20% to 60% in 60 min). BDNF was eluted as a major peak at about 45 min. This peak, containing about 20 µg BDNF, was evaporated to dryness, resuspended in 60 µl water and stored frozen until used. It was found to be maximally active (survival of chick dorsal-root sensory neurons on a laminin substrate⁹) at 200 pg ml⁻¹, and half-maximally at 80 pg ml⁻¹.

* Different from respective controls at $P < 0.01$ (Student's *t*-test).

cells located distally to that membrane. When these membranes are coated either with an extract from the neural tube¹⁵ or with laminin and BDNF¹⁶, neural-crest-derived cells distal to the membrane can be temporarily rescued. Finally, we know that the quantities of BDNF present in the central nervous system are extremely small (the purification factor necessary to achieve homogeneity is more than a million-fold⁸).

The effects of BDNF are not limited to neural-crest-derived neurons: placode-derived nodose ganglia were also found to contain increased numbers of neurons after injections of BDNF.

Table 2 Effects of BDNF on degenerating neurons

	Control	BDNF
Degenerating neurons	324 ± 64 (n = 7)	189 ± 27 (n = 6)*
Healthy neurons	6,405 ± 560 (n = 7)	6,017 ± 833 (n = 6)†

The 29th dorsal root ganglia (showing the sharpest peak of degenerating neurons according to ref. 18) were removed at day 6 and processed as above (Fig. 1). Degenerating neurons were counted in alternative sections under oil immersion at magnifications ×630 or ×1,000, using the criteria of Hamburger *et al.*⁶. BDNF-treated embryos were injected as above. The results are expressed as the means ± s.d. per ganglion.

* Different from control at $P < 0.01$ (Student's *t*-test).

† At this stage, the cumulative effects of neuronal death are not yet such that they would be expected to influence the total number of neurons after treatment with BDNF (see Fig. 1).

This was clearly not the case with NGF. The latter finding is in accord with what has been predicted from *in vitro* studies¹⁷.

It will be interesting to see in future studies which populations of central nervous system neurons can be affected by the addition of exogenous BDNF, either during normal development or after lesions. From *in vitro* work, we already know that most embryonic rat retinal ganglion cells can be rescued when cultured in the presence of BDNF¹⁹. These results, together with the data reported here, raise the possibility that BDNF could play an important role in the development of central sensory pathways.

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A neuronal mechanism for sensory gating during locomotion in a vertebrate

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The response of the foot to touch during walking depends on whether it is in the air or on the ground. In most animals, reflex responses to external stimuli are similarly adapted to their timing in the locomotor cycle, but there is only fragmentary information about the neural mechanisms involved. In arthropods, reflex modulation can occur in the sensory receptors themselves¹ and in neurons that discharge during locomotion^{2,3}. By recording with dye-filled microelectrodes from neurons in the spinal cord of frog embryos, we describe reflex modulation at the level of sensory interneurons. Sensory inputs from skin receptors excite a specific class of spinal sensory interneuron whose activity leads to reflex bending of the body away from the stimulus. During swimming, these inputs are gated by rhythmic postsynaptic inhibition, so that sensory drive reaches motor neurons only at phases in the locomotor cycle when the resulting contraction would likewise turn the embryo away from the stimulated side. Such gating of sensory pathways could be a general feature of all locomotor systems where responses to sensory stimuli need to be adapted to the phase of locomotion.

In immobilized *Xenopus* embryos, gentle stimulation of the trunk or tail skin evokes reflex responses where spinal motor neurons on the opposite side are excited and those on the same side are inhibited^{4,5}. In the mobile embryo, this avoidance reflex would cause the body to flex away from the stimulated side. The simplicity of the spinal cord of the embryo has enabled the major cell categories involved in the skin sensory pathways that

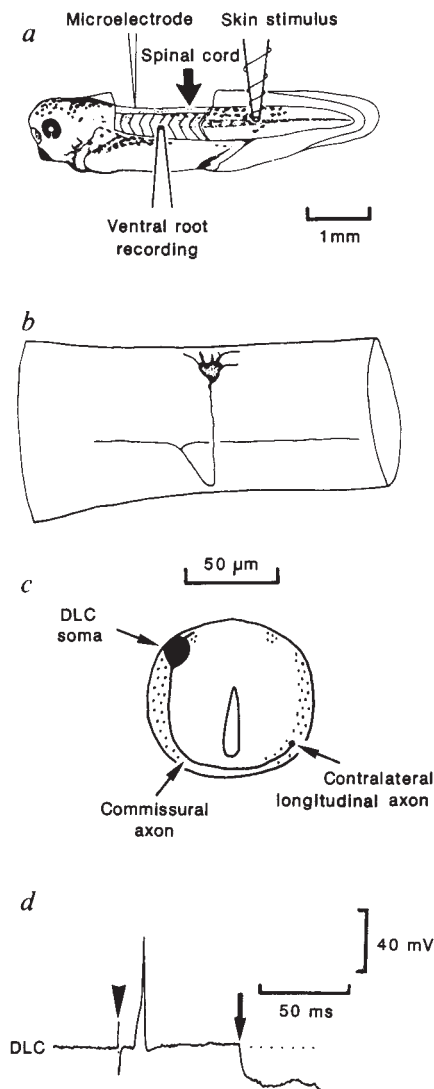


Fig. 1 Characterization of DLC sensory interneurons in the spinal cord. *a*, Scale drawing of *Xenopus laevis* embryo at stage 37/38 (ref. 26) showing locations of electrodes. Embryos were removed from egg membranes, paralysed in 100 μ M curare and dissected⁴. Intracellular recordings from 42 DL interneurons were made using glass microelectrodes filled with either 3 M potassium acetate (DC resistance 150–250 M Ω) or 5% Lucifer yellow²⁷ in 3 M lithium acetate (bevelled to $\sim$ 350 M Ω). *b*, Morphology of DLC interneuron revealed by iontophoretic injection of Lucifer yellow. Each of 9 DLC interneurons had dorsolateral, multipolar soma and axon which ran towards and, on 7 of 9 occasions, crossed ventral commissure. *c*, Schematic representation of DLC interneuron in cross-section, depicting major anatomical features. *d*, Physiology of DLC interneuron. In response to stimulation of skin on the same side as soma (arrowhead), each DLC interneuron received short latency ($\sim$ 7 ms) excitation and was inhibited at onset of swimming (arrow). Dots, resting potential.

initiate this reflex to be characterized⁶. Skin stimulation excites mechanoreceptive Rohon-Beard neurons⁷ that monosynaptically contact previously unidentified spinal interneurons^{4,8}. By recording intracellularly with Lucifer-yellow-filled microelectrodes (Fig. 1), we have now identified these interneurons. They are a distinct class of spinal sensory interneurons with multipolar somata and commissural axons projecting to the opposite side of the spinal cord: the dorsolateral commissural interneurons (DLC; Fig. 1*b*). Only two classes of spinal interneuron have commissural axons: DLC interneurons and commissural inter-

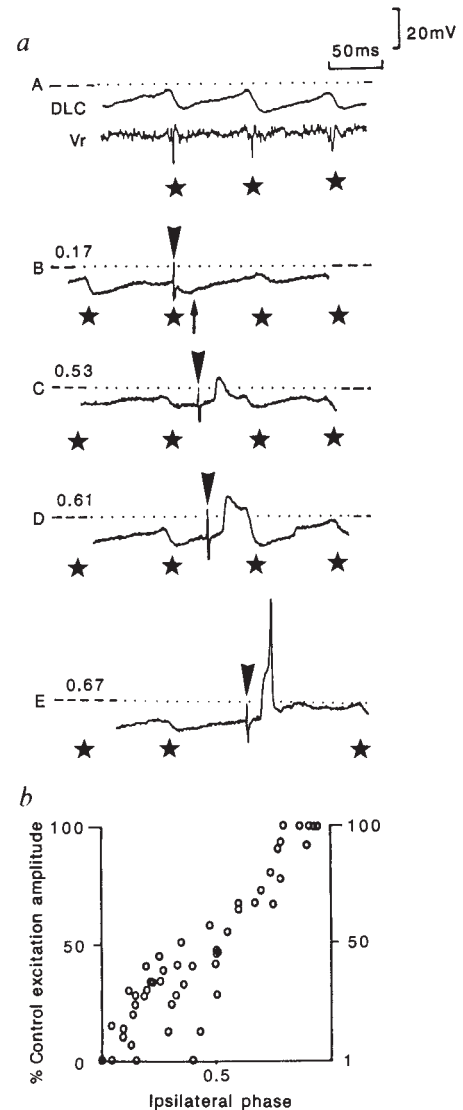
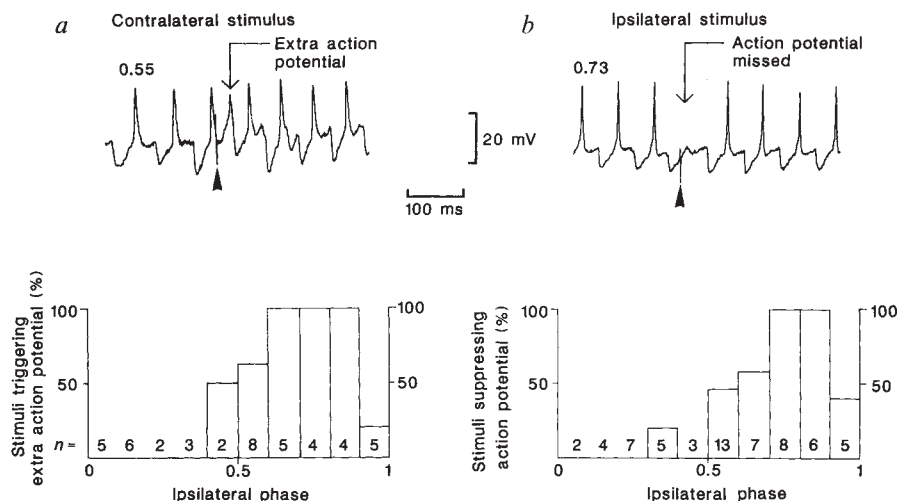


Fig. 2 Gating of sensory excitation in DLC interneurons during swimming activity. Panel *a*: A, during swimming a DLC interneuron (DLC, top trace) is rhythmically inhibited in phase with ventral root activity on same side (vr, lower trace, stars show timing of ventral root spikes); B–E, inhibition during swimming suppresses action potentials in a DLC interneuron. The ipsilateral skin was stimulated at an intensity that evoked an action potential and the stimulus repeated at 1 Hz during swimming. Stimuli (arrowheads) occurring early in a cycle evoked a very small excitation in the interneuron (B), whereas those occurring at later phases evoked larger excitation (C, D) and eventually evoked an action potential (E). Numbers show phase of response in ipsilateral cycle, stars show timing of ipsilateral ventral root spikes. Dots indicate resting potential. Panel *b*, excitation was evoked by ipsilateral skin stimulation. The stimulus was repeated at the same intensity throughout episodes of swimming. The amplitude of excitation was then measured as percentage of control (before onset of swimming) and plotted against phase relative to the ipsilateral ventral root. The 50 data points were taken from 25 episodes of swimming recorded in four interneurons.

neurons⁶. As the latter are inhibitory in action and contain glycine^{9,10}, we deduce that the source of excitation underlying the avoidance reflex is probably the DLC interneurons.

When released from their egg membranes near the time of hatching, *Xenopus* embryos will swim by lateral undulations of the body at frequencies of about 15 Hz. In immobilized embryos the basic alternating neural activity underlying swimming move-

Fig. 3 Phase-modulation of motor output during swimming following contralateral (*a*) and ipsilateral (*b*) skin stimulation. *a*, The contralateral skin was stimulated at an intensity that evoked excitation in a motoneuron and the stimulus repeated at 1 Hz during swimming activity: top, stimulus (arrowhead) at phase 0.55 (with respect to stimulated side) evoked an extra action potential; below, percentage of stimuli evoking extra action potentials versus ipsilateral phase. *n*, Number of stimuli in phase bin. Forty-four stimuli from 10 recordings. *b*, Effects of ipsilateral stimulation; same experimental procedures used as in *a*. Top, stimulus (arrowhead) at phase 0.73 (with respect to stimulated side) evoked inhibition that suppressed the action potential driven by the CPG for swimming; below, percentage ipsilateral stimuli suppressing action potentials versus ipsilateral phase. Sixty stimuli from 10 recordings. The lower percentage of stimuli triggering responses at phase values 0.9 to 1 are principally due to conduction delays.



ments is produced by the central nervous system¹¹. During fictive swimming, the nine dye-filled DLC interneurons that we recorded were rhythmically inhibited in phase with motor activity on the same side (Figs 1*d* and 2*a*, A). This suggested two simple and interrelated hypotheses⁴: firstly, the rhythmic inhibition could suppress or gate sensory excitation for part of each swimming cycle; secondly, if the DLC interneurons are responsible for the avoidance reflex, then the gating resulting from the inhibition should lead to a phase-dependent modulation of this reflex during swimming.

To test the first sensory-gating hypothesis, we stimulated the skin at an intensity that evoked excitation in a DLC interneuron, and related changes in the amplitude of the excitation to the phase of swimming. The excitation was powerfully suppressed early in each cycle when it coincided with the trough of inhibition (Fig. 2*a*, *b*), but at phase values of about 0.5 or more, little or no suppression occurred. Furthermore, if the stimulus was sufficient to evoke an action potential in the DLC interneuron, the inhibition blocked spiking activity for a similar portion of the swimming cycle (Fig. 2*a*). Thus, a rhythmic inhibition of the sensory DLC interneurons gates the flow of sensory information during swimming. The timing and strychnine sensitivity of the inhibition¹² strongly suggests that it comes from the ipsilateral axons of commissural interneurons, the reciprocal inhibitory premotor interneurons active during swimming⁹ that are thought to be part of central pattern generator (CPG) for swimming¹³.

To test the second reflex modulation hypothesis, we recorded intracellularly from motor neurons and evoked reflex excitation and inhibition by stimulating the contralateral and ipsilateral skin, respectively. The reflex responses were then observed during swimming (Fig. 3). Appropriately timed contralateral stimuli evoked excitation that could trigger an extra action potential in a motor neuron within the same cycle of swimming (Fig. 3*a*), whereas stimuli at other phases in a cycle did not evoke detectable excitation and the swimming continued apparently uninterrupted. The part of the cycle in which excitation could reach the motor neurons corresponded to phase-values of between 0.5 and 1—precisely the time when the gated sensory signal can be transmitted through the DLC interneuron pathway (Fig. 3*a*, lower panel, compare Fig. 2*b*). Reciprocal effects were also observed in motor neurons ipsilateral to the site of stimulation (Fig. 3*b*) where, at exactly the phase when contralateral motor neurons could be excited during swimming, ipsilateral motor neurons were inhibited, so that action potentials driven by the CPG for swimming were suppressed. The reciprocal reflex effects

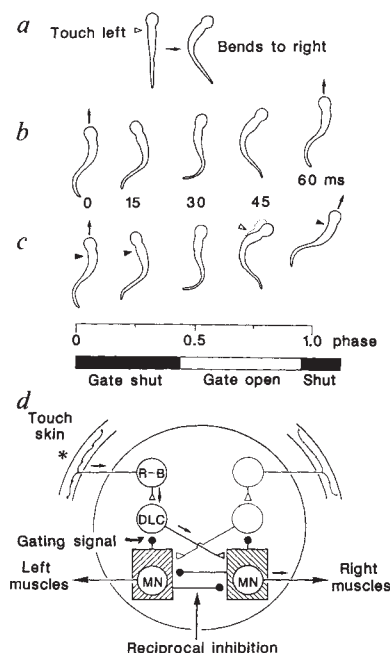


Fig. 4 The avoidance reflex (*a*), its gating during swimming (*b*, *c*) and the neural circuit responsible (*d*) in *Xenopus* embryos. *a*, In the avoidance reflex a touch to the left side of a quiescent animal (arrowhead) leads to reflex bending on the other side. *b*, Diagrams showing the embryo's movements during one 60 ms cycle of swimming. *c*, Diagrams to illustrate the expected effects of phase-dependent gating. Touch to the left side during swimming is ineffective when the body is bending to the left (black arrowheads, ipsilateral phase 0.9 to 0.4) and is only effective when the body is bending to the right (for example, open arrowhead, ipsilateral phase 0.5 to 0.9). Enhanced contraction on the opposite side would change the direction of swimming (arrows) away from the side touched. *d*, The neural circuit for the reflex and sensory gating shown in a schematic section of the spinal cord. Touching the skin on the left side (*) excites sensory neurons (R-B). These excite sensory interneurons (DLC) which cross the cord to excite neurons of the motor system (hatched) including motoneurons (MN) on the right side. Inhibition is sent back to the left side by the reciprocal inhibition pathway. During swimming, rhythmic inhibition from the motor system on the same side (hatched) to DLC sensory interneurons (gating signal) gates the flow of excitation from sensory R-B cells to motoneurons on the other side (small arrows). Circles, populations of neurons; open triangles, excitatory synapses; small dark circles, inhibitory synapses.

of sensory stimulation observed in quiescent animals are thus preserved by the sensory gate so that reflex excitation of motor neurons on one side is reinforced by simultaneous inhibition of antagonists on the opposite side. The effect of these phase-modulated reflexes is that a stimulus can only excite the opposite side when it is already contracting. This results in enhanced contraction on the opposite side which would direct swimming away from the side stimulated, just as the avoidance reflex in the quiescent animal bends the body away from the stimulus (Fig. 4).

Central modulation of reflexes during locomotion, like that described here, is a general feature of invertebrate^{1-3,14,15} and vertebrate¹⁶⁻²⁴ motor systems. It is assumed that such modulation could result if the CPGs for locomotion modified the input-output properties of central nervous pathways according to phase. This could occur at four principal levels: (1) the central terminals of sensory receptors, (2) the sensory interneurons that they excite, (3) premotor interneurons that are rhythmically active during locomotion and (4) the motor neurons that they drive. Rhythmic primary afferent depolarization during fictive locomotion is present in cats²⁵ and underlies the presynaptic modulation of sensory transmission (level 1) in the crayfish¹. Our study of swimming in *Xenopus* embryos has revealed a mechanism whereby postsynaptic inhibition controls the flow of sensory information through sensory interneurons which are not active during locomotion (level 2). A rhythmic, inhibitory gating signal from the CPG for swimming suppresses sensory transmission in a phase-dependent manner which would result in the embryo turning away from the stimulated side. During locust flight the alternating inhibitory and excitatory synaptic drive that underlies rhythmic firing of motor and premotor neurons (levels 3 and 4) has been shown to modulate sensory feedback. Descending information signalling deviation from the flight path is transformed into a phase-dependent reflex which corrects for flight instability^{2,3}. Thus, evidence on mechanisms for reflex modulation now exists at all four levels, in the nervous systems of simple preparations. The striking parallels between the frog embryo and locust suggest that the generality of these mechanisms now needs to be tested by experiments on higher vertebrates.

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A suppressor T-lymphocyte cell line for autoimmune encephalomyelitis

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Experimental allergic encephalomyelitis (EAE) is a model for the *in vitro* and *in vivo* study of T-cell activation. It is an autoimmune disease mediated by T lymphocytes of the helper T-cell (T_H) subset^{1,2}. After sensitization to guinea-pig myelin basic protein in complete Freund's adjuvant, Lewis rats develop an autoimmune response to central nervous system (CNS) myelin basic protein, manifested clinically as paralysis and histologically by a perivascular mononuclear cell infiltrate of the CNS parenchyma. Suppressor cell regulation of EAE has long been suspected because Lewis rats, which spontaneously recover from active disease, are resistant to reinduction of active EAE, even though effector T-cell lines can be rescued from these recovered rats³. Using cyclosporin A, an immunosuppressive agent believed to inhibit T_H cell function, suppressor T-cell (T_S) lines have now been generated from recovered Lewis rats. These T_S cells, when admixed with guinea pig myelin basic protein-specific T_H cells, will prevent the adoptive transfer of EAE. The T_S cells appear to be $CD4^+$, which explains previous observations that $CD8^+$ lymphocytes are not important in the recovery of EAE in the rat. This is the first direct demonstration of T_S -cell regulation of EAE.

Lewis rats which have recovered from EAE are resistant to reinduction of active disease by day 30 post-immunization⁴. Therefore, T_S -cell lines were generated from the draining lymph nodes of animals immunized one month beforehand with guinea pig myelin basic protein (GP-MBP) in complete Freund's adjuvant (CFA). To select for T_S cells rather than T_H cells, the lymph node cells were grown for seven days in the presence of GP-MBP and cyclosporin A. The resulting blasts were purified and grown for 1-7 days in the presence of supernatants from concanavalin A-stimulated cells as a source of growth factors. The cells were maintained in culture by alternate activation with GP-MBP and a source of antigen-presenting cells, followed by growth in concanavalin A-stimulated cell supernatants, as described in Table 1. The T_S -cell lines thus generated were specific for GP-MBP, but not for PPD (the immunogenic moiety of CFA), or for bovine P_2 protein⁵, a peripheral nervous system myelin basic protein.

After 15 cycles of antigenic stimulation, the T-cell lineage of one such functionally mature cell line was confirmed by cell-surface marker phenotyping with monoclonal antibodies raised against rat T-cell differentiation antigens⁶⁻¹¹. The cell line CsLN-1 reacted with MRC OX19, a rat pan T-cell ($CD5$) marker. Virtually all (>99%) of CsLN-1 reacted with two different rat anti- $CD4$ monoclonal antibodies against non-competing epitopes (MRC OX35 and W3/25), and were strongly $CD4^+$ in both the activated and resting states. The cell line did not express major histocompatibility complex class II I-A or I-E antigens. Rat $CD4$ (W3/25)⁺ suppressor T cells are not without precedent: W3/25⁺ T cells effect partial¹² or nearly complete suppression¹³ of allograft rejection in two different rat transplantation models. There have been suggestions that $CD4^+$ suppressor cells may function in immunoregulation of rat EAE (ref. 14), based on the observation that $CD8^+$ cells are not apparently important in the recovery from and resistance to subsequent episodes of rat EAE (ref. 15). The present description of the isolation of a $CD4^+$ suppressor cell from recovered EAE rats would be consistent with those observations.

It is interesting to note that although CsLN-1 is $CD8^-$, ~20-30% of the functionally mature cells (11-15 cycles of antigenic

Table 1 Antigen specificity of the *in vitro* proliferation (c.p.m.) of CsLN-1

Antigen	Cycles of stimulation		
	5×	8×	11×
None	24,439	19,416	448
GP-MBP	193,894	202,413	64,938
PPD	13,374	5,428	NT
Concanavalin A	216,951	133,610	66,929
P ₂	NT	NT	228

To generate T_s cell lines, female Lewis rats, 8–12 weeks of age, were immunized in both hind foot pads with 50 µg GP-MBP emulsified in CFA. One month post-immunization, the draining lymph nodes were removed, processed into a single-cell suspension, and washed once in Dulbecco's A+B medium containing 0.2% bovine serum albumin. Lymph node cells were plated at 5×10^6 ml⁻¹ in six-well clusters (Costar 3406) in RPMI 1640 (Gibco), 1% Lewis rat serum, 200–300 ng ml⁻¹ cyclosporin A (Sandimmune IV, Sandoz), 50–60 µg ml⁻¹ GP-MBP at 37°C in 5% CO₂. After 7 d, the activated blasts were purified on Ficoll-Metrizoate (density 1.077 g ml⁻¹), and plated at 2×10^5 ml⁻¹ in RPMI 1640, 10% heat-inactivated fetal calf serum (Hyclone), and 10% supernatant from concanavalin A-stimulated cells (derived from 48 h-concanavalin A-activated Lewis rat splenocytes). After 1–7 days, the cells were re-stimulated for 3 d at 3×10^5 ml⁻¹ with GP-MBP (50 µg ml⁻¹) and 1×10^7 ml⁻¹ irradiated Lewis thymocytes (2,500 R) in RPMI 1640, 1% Lewis rat serum. Thereafter, cell lines were alternately passaged as above in 10% supernatant from concanavalin A-stimulated cells or with antigen and antigen-presenting cells. Complete culture media consisted of RPMI 1640, supplemented with 1mM sodium pyruvate, 2mM glutamine, 10mM HEPES buffer, 5×10^{-5} M2-mercaptoethanol, 100 U penicillin per ml, 100 µg streptomycin per ml, and 25 ng Fungizone per ml (Gibco). To test for antigen specificity, CsLN-1 was plated in RPMI 1640 with 1% Lewis rat serum at 4×10^4 cell per well with 1×10^6 per well irradiated (2,500 R) Lewis thymocytes in 96-well flat-bottomed trays (Costar) at 0.2 ml final volume. After 48 h, cultures were pulsed with 0.5 µCi ³H thymidine and cells were collected 16 h later. Incorporation of ³H thymidine was measured by liquid scintillation spectroscopy. Results are expressed as c.p.m. of four replicates. Standard errors were within 20% of the mean and are not shown. GP-MBP, PPD and P₂ were used at 25 µg ml⁻¹; concanavalin A was used at 2.5 µg ml⁻¹; NT, not tested.

stimulation) become transiently CD8⁺ on stimulation with concanavalin A or antigen, but expression is at a lower level compared with CD4. In contrast, the expression of CD4 is stable and comparable in both resting and activated cells. The activation-dependent co-expression of CD4 and CD8 cell-surface markers has been described for both human and rat polyclonal T-cell populations^{16–18}. Human T_s clones co-express the CD4 and CD8 antigens¹⁹. This phenomenon has also been observed with at least one GP-MBP-specific T_h cell clone that produces EAE in the rat. Some 40% of the cells in a CD4⁺ T-effector cell clone (H. Offner and A. Vandenbark, personal communication) co-express CD8⁺ on antigen activation. Although the function of the CD8 marker on CsLN-1 is unclear, they do not appear to be important in the suppressive activity of CsLN-1. The continuous presence of anti-CD8 (MRC OX8) at 10 µg ml⁻¹ in culture during both the activation of CsLN-1 with antigen, and the subsequent co-culture of CsLN-1 with splenocytes from GP-MBP-sensitized animals, repeatedly had no effect on the ability of the CsLN-1 to suppress the transfer of EAE. The presence of MRC OX8 by itself does not affect the transfer of EAE (ref. 2). The purpose of the co-expression of CD8 with CD4 during activation is therefore not clear.

As EAE T_h-effector cell lines are also CD4⁺, CsLN-1 was tested for its ability to passively transfer EAE to naïve rats. Table 2 shows that after 5–6 cycles of antigenic stimulation, CsLN-1 was able to produce mild clinical signs of disease in each of four rats. The severity and incidence of passive disease produced by CsLN-1 decreased with subsequent cycles of antigenic stimulation, until CsLN-1 was no longer able to produce

Table 2 Ability of activated CsLN-1 cells to produce EAE in Lewis rats as a function of cycles of stimulation

Activation cycle	Stimulus	Maximum disease severity			
		0	1	2	3
5×	GP-MBP	—	3	1	—
6×	GP-MBP	—	3	1	—
7×	GP-MBP	3	1	—	—
8×	GP-MBP	3	1	—	—
9×	GP-MBP	4	—	—	—
9×*	concanavalin A	4	—	—	—
11×	concanavalin A	4	—	—	—

CsLN-1 at 2×10^5 ml⁻¹ were grown in RPMI 1640, 1% Lewis rat serum, with irradiated (2,500 R) Lewis thymocytes at 1×10^7 ml⁻¹ and GP-MBP at 20–25 µg ml⁻¹ or concanavalin A at 5 µg ml⁻¹. After 3 d at 37°C in 5% CO₂, cells were collected and 5×10^6 activated cells were injected intraperitoneally into each of four naïve Lewis rats in 2.0–3.0 ml PBS. The appearance of clinical signs of EAE (~4–6 days post-transfer) was graded on a three-point scale as follows: 1, limp tail; 2, hind leg weakness; 3, hind leg paralysis.

* Each rat received 2×10^7 concanavalin A-activated CsLN-1.

Table 3 Effect of T_s-cell lines on the adoptive transfer of EAE

	Incidence of EAE	Mean severity (max. 3.0)	Mean histology (max. 3.0)
Activated CsLN-1	0/12	0	0.6 (n = 7)
Resting CsLN-1	1/3	0.33	1.5 (n = 3)
Activated P ₂ T-cell line	7/7	2.2	NT
Controls	15/15	2.7	2.6 (n = 7)

CsLN-1 was activated *in vitro* for 3 d with GP-MBP and APC as described in Table 2. The P₂T-cell line was activated in culture at 2×10^5 cells per ml with bovine P₂ at 40 µg ml⁻¹ and APC for 3 d. Spleen cell donors were female Lewis rats immunized 11–12 days earlier with 50 µg GP-MBP in CFA. GP-MBP-sensitized spleen cells were plated at 2×10^6 ml⁻¹ in complete culture media supplemented with 5% heat-inactivated fetal calf serum, and GP-MBP at 2 µg ml⁻¹ in 80 cm² tissue-culture flasks. To test for suppression, CsLN-1 or the P₂ T-cell line were added at a ratio of one T_s cell to eight spleen cells. Cells were cultured for 3 d at 37°C in 5% CO₂. Collected cells were injected i.p. into naïve Lewis rats. Recipients of control cells were given $3–4 \times 10^7$ viable spleen cells; recipients of spleen cells mixed with T_s cells were given 10^7 more viable cells than matched controls. Clinical signs of disease was graded on the three-point scale shown in Table 2. Cryostat sections of brain and spinal cord were taken on days 12–14 after cell transfer, stained with haematoxylin and eosin, and graded on the following scale: 0, no abnormality; 1, an occasional foci of perivenular leukocytes within the neuraxis; 2, many small foci of perivenular leukocytes within the neuraxis; 3, large and numerous foci of perivenular leukocytes within the neuraxis.

any clinical signs of EAE. Activated cells were tested for effector activity at a dose of 5×10^6 cells per recipient because each of four extant EAE T_h-cell lines transfer paralytic disease at that cell number^{20–23}. In fact, two of the four effector cell lines are lethal at that dose. It was concluded that CsLN-1 was not an EAE-producing T_h-cell line. Some EAE T_h cells were included, however, in the initial population of CsLN-1. These T_h cells were either lost or their activity was suppressed with time.

CsLN-1 was assayed for suppressive activity by using the adoptive transfer model of EAE (ref. 24). Adoptive transfer of EAE can be achieved with spleen cells taken from GP-MBP-sensitized Lewis rats on day 11 or 12 post-immunization and activated *in vitro* with antigen. Recipients of $3–4 \times 10^7$ activated splenocytes develop severe paralytic EAE 4–6 days later. As shown in Table 3, recipients of splenocytes mixed with GP-MBP-activated T_s cells during the *in vitro* antigen activation period, showed no clinical evidence of disease after transfer of up to 5×10^7 cells. Histological evidence of disease was seen only if

the T_s cells were not antigen-activated before the 3-day culture period with sensitized spleen cells, or if the T_s -cell line was used before all T_h effector activity had disappeared from the line (as shown in Table 2). This effect was not merely due to the competition of activated T cells for growth factors in the media, because an activated P_2 T-cell line (T. Olee and S.W.B, unpublished) did not show the same suppressive effect when co-cultured *in vitro* with GP-MBP-sensitized spleen cells and antigen. The results in Table 3 shows the ability of these T_s cell lines to prevent EAE transfer and represents the first isolation of a distinct population of cells with suppressor activity for this model of T-cell activation.

The cell lines were tested for any effects on active EAE. $0.5-2 \times 10^7$ GP-MBP-activated cells were injected into Lewis rats at various times before or after sensitization with GP-MBP in CFA. None of the protocols was able to prevent the development of active disease or reduce its severity. The failure of the cell lines to act *in vivo* to block the induction of EAE could be due to the different migratory patterns of activated T cells. Activated murine T cells are unable to traffic to peripheral lymphoid organs after injection into syngeneic recipients²⁵. This is because activated T lymphoblasts lack a cell-surface receptor for attachment to the high endothelial venules of peripheral lymphoid tissue, which then allows their egress from the circulation into the lymphoid parenchyma. Presumably, a T_s cell would need to home to the draining lymph nodes of a GP-MBP-immunized animal to effect suppression of disease. Unlike the active model of EAE, the CsLN-1 cells in the transfer model of disease are placed in direct contact with the effector cells during antigen activation, ensuring interaction between the effector cells and putative suppressor-cell line, which might not occur in the *in vivo* model of the disease.

Isolation of a $CD4^+$ T-cell line with suppressive activity for EAE could permit a better understanding of how autoimmune T-cell-mediated diseases can be down-regulated. If the activation mechanism of these cells is understood, be it by direct contact and/or by the release of suppressor factors, a therapy for human autoimmune disease might follow.

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Expression of functional interleukin-2 receptors in human light chain/Tac transgenic mice

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The growth of mature T lymphocytes is regulated by interaction between interleukin-2 (IL-2) and its receptor¹. Three distinct binding sites for IL-2, namely low- (K_d 10 nM), intermediate- (K_d 100 pM) and high- (K_d 10 pM) affinity sites, have been found on human and primate T lymphocytes²⁻⁴. Chemical crosslinking of labelled IL-2 to human T cells shows that two polypeptide chains, p55 (L chain) and p75 (H chain), bind IL-2 with low and intermediate affinities respectively. The high-affinity binding was shown to arise from ternary complex formation of IL-2, L and H chains⁵. Construction of mutants of the L-chain complementary DNA indicated that the L chain is not directly involved in growth signal transduction⁶. Nevertheless, expression of the IL-2 receptor L chain is tightly regulated by antigen or mitogen stimulation. To investigate the L chain function, we have produced transgenic mice using human L-chain cDNA of the IL-2 receptor under the control of a constitutive promoter. Studies on the L-chain transgenic mice showed that functionally active IL-2 receptors with high affinity were expressed on unstimulated spleen and thymus cells. The results indicate that the H chain of the IL-2 receptor is constitutively expressed in T cells.

L-chain cDNA (ref. 7) of the human IL-2 receptor was fused to the mouse H-2K^d (MHC class I molecule) promoter⁸. The construct (Fig. 1a) carrying the complete L-chain cDNA of the human IL-2 receptor was introduced into fertilized mouse eggs. We obtained four founder animals containing the human L-

chain cDNA in the germline and analysed one (R5) of the strains in detail, but the others showed similar properties. Expression of human L-chain messenger RNA was analysed using a ribonuclease protection assay (Fig. 1b). Maximal expression of human L-chain mRNA was observed in spleen and thymus; decreased, but readily detectable, expression was found in liver. Brain and kidney contained almost no human L-chain mRNA. On the other hand, endogenous mouse L-chain mRNA was detected only in thymus and not in spleen, in agreement with a previous report^{9,10}. Direct immunofluorescent staining profiles with antibody raised against human L chain (anti-Tac) (ref. 11) showed that the human L chain was expressed strongly on spleen cells and to a lesser extent on thymocytes (Fig. 2a, b).

We studied the kinetics of binding of human recombinant IL-2 to spleen cells of transgenic mice^{12,13}. As shown in Fig. 3, spleen cells of transgenic mice had ~120 high-affinity sites (K_d 26 pM) and 800 low-affinity sites (K_d 1 nM) per cell. We could not detect binding of IL-2 to spleen cells of normal mice (data not shown). IL-2 binding to spleen cells of transgenic mice was blocked completely by anti-Tac antibody, but was not affected by anti-mouse IL-2 receptor (L-chain) antibody PC61 (ref. 9). The results indicate that the high-affinity binding of IL-2 is due to expression of exogenous human L chains.

As shown in Table 1, when added to a culture of transgenic spleen cells, IL-2 induced dose-dependent growth response. Addition of anti-Tac antibody to this culture inhibited the response, whereas PC61 and a myeloma protein HOPCL did not influence the growth of the spleen cells. As previously reported for human lymphocytes¹⁴, normal murine spleen cells also grew, albeit very weakly, in response to IL-2, and their growth was inhibited by PC61. The results are evidence for the constitutive expression of functional H chains in spleen cells.

We then purified non-T spleen cells (Thy 1.2⁻) by treatment of whole spleen cells with a monoclonal anti-Thy 1.2 antibody plus complement. The Thy 1.2⁻ cells did not respond to concanavalin A. These cells were stained by the anti-Tac antibody

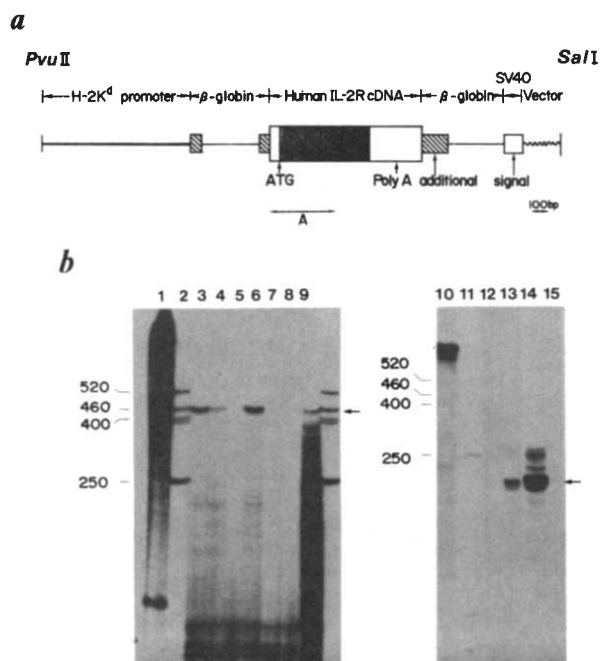


Fig. 1 *a*, Structure of L chain cDNA of the human IL-2 receptor in expression vector. The plasmid pKCR·H-2K^d·h·IL-2R was constructed by replacing the promoter part of pKCR·Tac 2A (ref. 7) with a fragment of H-2K^d promoter⁸. The *Pvu*II-*Bam*HI fragment of the H-2K^d promoter (the *Bam*HI cleavage site was 13 base pairs upstream of the initiation codon) was inserted into *Pvu*II-*Bam*HI site of pKCR·Tac 2A. The plasmid was linearized by digestion with *Pvu*II and *Sal*I and injected into fertilized one-cell embryos of C57BL/6 mice, which were implanted into pseudopregnant female ICR mice and allowed to develop. DNA was adjusted to 10 ng μ l⁻¹ in 10 mM Tris-HCl (pH 8.0), 0.2 mM EDTA; microinjection, embryo transfer and DNA analysis are described elsewhere¹⁹. Fragment A (440 base pairs), indicated by a horizontal arrow, was subcloned in vector Sp6 and used for making the anti-sense RNA probe for the ribonuclease protection assay. *b*, RNA transcripts of the human L chain transgene. RNAs from tissues of transgenic mice were analysed by the ribonuclease protection assay using two probes: one (lanes 1-9) hybridizable to the first 440 bases of human L chain mRNA, and the other (lanes 10-15) hybridizable to 190 bases of murine L chain mRNA. RNAs used are: 1 and 10, RNA without RNase treatment; 2 and 11, size markers; 3 and 12, from spleen; 4, from liver; 5, from kidney; 6 and 13, from thymus; 7, from brain; 8 and 14, RNA from a murine T-cell line CTLL-2 (ref. 20); 9 and 15, RNA from a human T-cell line MT-1 (ref. 21).

Methods. RNA was isolated from the individual organs as described²². RNA (20 μ g) was hybridized overnight at 50 °C with a ³²P-labelled anti-sense *in vitro* transcript of human or murine L chain, followed by digestion with ribonuclease A and T₁ as described²³. The protected fragments were separated on a 4% polyacrylamide/urea gel and exposed to an X-ray film.

in the same way as whole spleen cells (Fig. 2c, d, e), but they responded to IL-2 much more feebly (Table 1). The results indicate that the H chain is expressed predominantly in T cells.

As previously reported, ~50% CD4(L3T4)⁺/CD8(Lyt2)⁻ cells in thymus express receptors for IL-2, but they do not grow in IL-2-containing media at all or respond to IL-2 only weakly^{9,10,15}. The results indicate that thymocytes expressing the endogenous L chain do not express the H chain. But the transgenic thymocytes grew in response to IL-2 (Table 1). Such proliferative potential of transgenic thymocytes could arise from almost or fully mature T cells contaminating our thymocyte preparations. Alternatively, there might be another subset of thymocytes that express only the H chain constitutively.

The present study demonstrates that the human IL-2 receptor L chain expressed in T cells of transgenic mice can act as a functional IL-2 receptor without stimulation, indicating that the

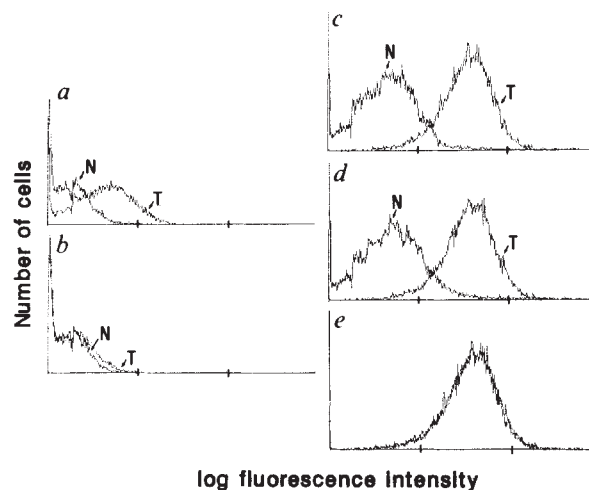


Fig. 2 Flow cytometric analysis of human IL-2 receptor expression by spleen cells and thymocytes of transgenic mice. Spleen cells and thymocytes of transgenic and normal mice were stained with anti-Tac monoclonal antibody as described below. *a*, Whole spleen cells prepared for experiment 1 in Table 1. *b*, Whole thymocytes prepared for experiment 1 in Table 1. *c*, Whole spleen cells prepared for experiment 2 in Table 1. *d*, Thy 1.2⁻ spleen cells prepared for experiment 2 in Table 1. *e*, Comparison between whole and Thy 1.2⁻ spleen cells of transgenic mice. The profiles shown in *c* and *d* were superimposed. N, Cells from normal control mice. T, Cells from transgenic mice.

Methods. In *a* and *b*, spleen cells and thymocytes were obtained and red cells were lysed by adding an equal volume of 1.66% NH₄Cl. Nucleated cells were then washed twice and cell suspensions (5 × 10⁶ cells in 50 μ l) were incubated in Hanks solution containing 1% fetal calf serum (FCS) and 0.1% NaN₃ with FITC-labelled anti-Tac antibody for 30 min at 4 °C. Cells were washed three times, resuspended in 1 ml Hanks-FCS-NaN₃ solution and assayed by fluorescence-activated cell sorter (EPICS V, Coulter). In *c*, *d* and *e*, spleen cells prepared as above were treated further: cells (1.2 × 10⁶ ml⁻¹) in cytotoxicity medium (RPMI 1640, 0.3% BSA, 25 mM HEPES) were incubated with (for Thy 1.2⁻ spleen cells) or without (for whole spleen cells) 40 μ g ml⁻¹ mouse IgM monoclonal anti-Thy 1.2 antibody (Miles) for 45 min at 4 °C. Then cells were washed once and resuspended in 2 ml cytotoxicity medium containing 1/10 dilution of rabbit complement (Cedarlane). After incubation for 45 min at 37 °C, cells were washed twice and cell suspensions (5 × 10⁶ cells in 50 μ l) were incubated in the Hanks-FCS-NaN₃ solution with 50 μ g ml⁻¹ biotinylated anti-Tac antibody for 50 min at 4 °C. The cells were then washed three times and resuspended in 100 μ l of avidin dilution buffer (0.15 M NaCl, 10 mM HEPES, 0.07% NaN₃) containing 12.5 μ g ml⁻¹ FITC-avidin (Vector Laboratory). Following incubation for 30 min at 4 °C, the cells were washed three times, resuspended in 1 ml of the Hanks-FCS-NaN₃ solution and analysed as in *a* and *b*.

H chain of the IL-2 receptor is constitutively expressed in spleen and thymus. It is unlikely that the expression of the H chain is induced by the transgenic expression of the L chain, because some thymocytes express the L chain alone, as described above. Thy 1.2⁻ cells of transgenic mice also grew in IL-2-containing media, although very weakly. It is not clear whether or not non-T cells responding to IL-2 are B cells or large granular lymphocytes which can express the H chain^{16,17}. All transgenic mice appear healthy, probably because the human IL-2 receptor does not respond to murine IL-2.

Why are the two chains of the IL-2 receptor regulated in different ways? The H chain seems to be responsible for growth signal transduction, in which the L chain *per se* is not directly involved⁶. It is possible that the H chain contains the kinase domain like other growth factor receptors. Because the amplification of oncogenes encoding the kinase domain causes cell transformation¹⁸, expression of limited and stable numbers of H chains could be important. Nevertheless, lymphocytes

Table 1 Human IL-2-induced proliferation of spleen cells and thymocytes from transgenic mice

IL-2 (pM)	Addition	[³ H]Thymidine incorporation (c.p.m.)							
		Experiment 1				Experiment 2			
		Spleen cells	Thymocytes	Whole spleen cells	Thy 1.2 ⁺ spleen cells	Transgenic	Normal	Transgenic	Normal
0	none	240	280	1,160	660	300	560	340	790
7	none	2,250	360	3,540	1,130	2,250	560	1,380	1,020
70	none	9,560	620	17,190	1,710	5,790	1,020	1,850	1,060
700	none	11,730	770	22,060	2,030	7,230	1,520	2,130	1,410
700	anti-Tac	770	700	3,410	1,920	970	1,230	810	1,110
700	HOPCI	10,070	690	ND	ND	6,360	1,430	2,030	1,330
700	PC61	10,510	310	ND	ND	7,440	920	2,260	1,090
0	Con A	ND	ND	ND	ND	33,260	45,920	170	270

Cell preparations are described in the legend to Fig. 2. Standard cultures in flat-bottomed microtitre wells contained 1×10^5 spleen cells or 5×10^5 thymocytes in 0.2 ml RPMI 1640 medium containing 10% fetal calf serum (FCS), 50 μ M 2-mercaptoethanol, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and IL-2 as indicated. After culture for 38 h at 37 °C in 5% CO₂, 0.5 μ Ci [³H]thymidine (Amersham, 2.0 Ci mmol⁻¹) was added to each well, and further incubated for 10 h. Cells were collected using a microharvester and acid-insoluble radioactivity was counted by liquid scintillation counting. The final concentrations of antibodies and concanavalin A (con A) were as follows: anti-Tac for spleen cells, 12.5 μ g ml⁻¹; anti-Tac for thymocytes, 100 μ g ml⁻¹; HOPCI, 12.5 μ g ml⁻¹; PC61, 160-fold dilution of ascites fluid; Con A, 5 μ g ml⁻¹. Data are means of triplicate experiments. The s.e.m. in these experiments was less than 10%. ND, not determined.

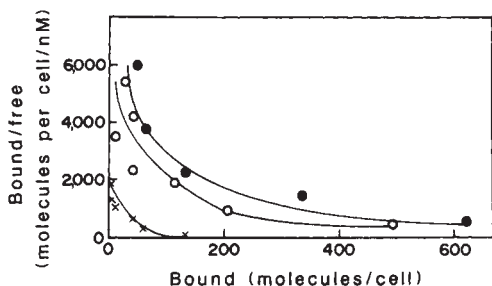


Fig. 3 Scatchard plot analysis of equilibrium binding of human recombinant ¹²⁵I-labelled IL-2 to spleen cells from a transgenic mouse. ●, Transgenic spleen cells; ○, transgenic spleen cells in the presence of PC61; x, transgenic spleen cells in the presence of anti-Tac.

Methods. Aliquots (200 μ l) of 2×10^6 cells were incubated for 30 min at 37 °C with various concentrations of ¹²⁵I-labelled IL-2. The specific activity and relative molecular mass of ¹²⁵I-labelled IL-2 were 30,000 c.p.m. ng⁻¹ and 15,000, respectively. Free and cell-bound IL-2 were separated by centrifugation through an olive oil cushion and measured using a γ -counter. The extent of non-specific binding was measured by incubating the cells in the presence of a 100-fold excess of unlabelled IL-2, and subtracting this value from the bound counts. IL-2 was labelled with ¹²⁵I according to ref. 24. PC61 antibody completely blocks binding of human IL-2 to the murine IL-2 receptor^{6,13}.

require a large number of IL-2 receptors to capture IL-2 at low concentrations for efficient cell growth. Induction of a large number of L chains inert to cellular activity yet efficient for binding IL-2 by high-affinity site formation could be a strategy for lymphocytes to avoid transformation while transducing growth signal efficiently.

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Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens

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Primary infection with human cytomegalovirus (HCMV) is persistent and widespread, with symptoms that are mostly subclinical but can cause serious illness or death, particularly in immunosuppressed patients^{1,2}. Recently³, proteins from HCMV were shown to bind β_2 -microglobulin (β_2 -m) a protein that is normally found associated with the class-I major histocompatibility complex (MHC) antigens, which are essential for self-non-self recognition in the immune response⁴. These findings led to the proposal that the virus may use β_2 -m binding as an infection mechanism⁵. Here we present evidence from DNA sequence analysis that HCMV encodes a molecule similar to the MHC class-I antigens of higher eucaryotes, and propose that this protein is responsible for the observed β_2 -m binding. The deduced amino-acid sequence of the HCMV class-I-like protein reveals conservation of typical features of class-I structure, but we predict that the gene is not spliced, in contrast to the cellular genes.

MHC class-I α chains are highly polymorphic and are found associated non-covalently with β_2 -m. They are encoded by eight exons separated by seven introns and show a very conserved

Fig. 1 Physical map of HCMV strain AD169. The linear double-stranded DNA genome of HCMV consists of a long unique (U_L) and a short unique (U_S) region, each flanked by repeat sequences (boxed) which allow the virus to exist in four equimolar isomers¹⁶. The scale shows the genome size in kilobases (kb). Below the scale the *Hind*III map of the generally accepted prototype orientation is shown. The *Hind*III O-fragment is indicated by shading and the approximate position of the predicted HCMV-H301 gene is arrowed.

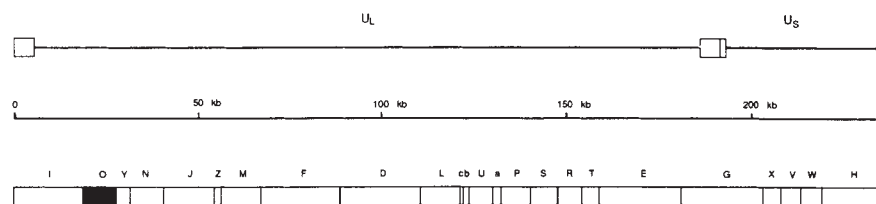
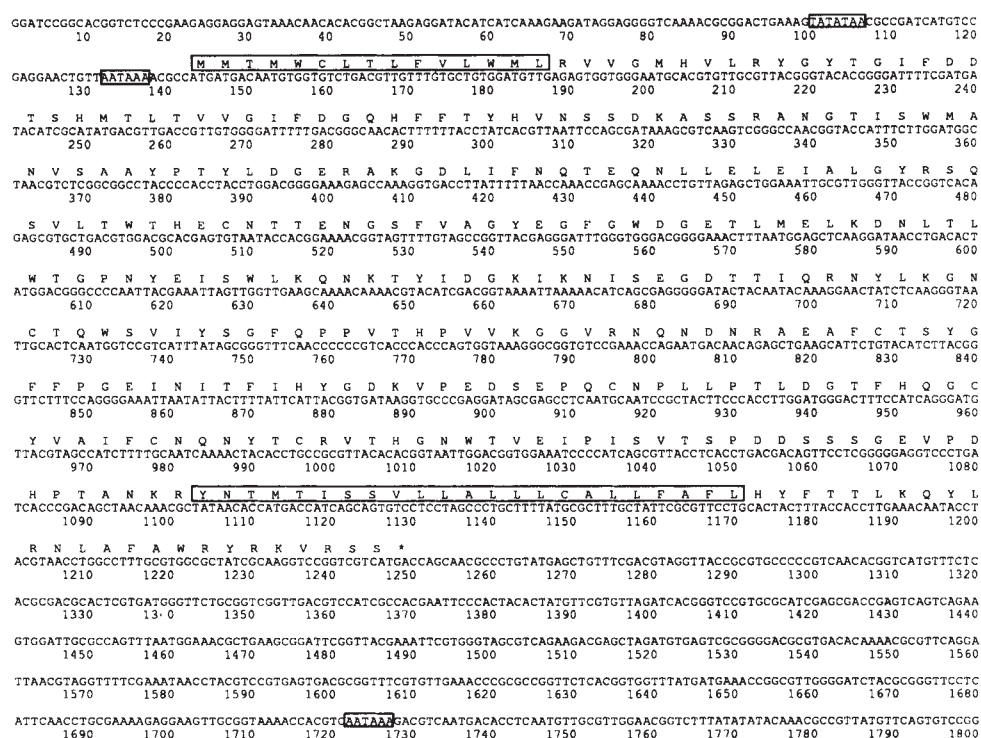


Fig. 2 Nucleotide sequence of the HCMV-H301 gene in 5'-3' direction. The predicted coding sequence starting at position 143 (start codon, ATG) to position 1,247 (stop codon, TGA) has been translated into the corresponding amino-acid sequence which is written above the nucleotide sequence in the one letter code. The potential TATA Box (position 100) at -42 upstream of the start codon and the potential polyadenylation site AATAAA (position 1,722) at +473 downstream of the stop codon are indicated by boxing. A second polyadenylation site (position 132) of an upstream open reading frame, ending at position 32 (stop codon, TAA), is also indicated by boxing. A third, short open reading frame downstream of the HCMV-H301 gene is present from position 1,246 (start codon, ATG) to position 1,540 (stop codon, TGA). The potential leader sequence (LD) and the transmembrane region (TM) of the deduced amino-acid sequence are also boxed in. The nucleotide sequence was determined on both strands according to the M13 shotgun dideoxy-chain termination technique^{17,18} and was analysed using the Staden software¹⁹.



gene structure^{4,6}. Analysis of the viral class-I-like gene (HCMV-H301) shows no suitable GT/AG splicing signals and the predicted coding sequence of 1,104 base pairs (bp) coincides with the average length of the coding sequences (about 1,100 bp) of known class I genes. We therefore assume that the HCMV gene is not spliced. The approximate position of the viral class-I-like gene in the HCMV genome is shown in Fig. 1, and part of the nucleotide sequence of the *Hind*III O-fragment encoding HCMV-H301 is shown in Fig. 2. The predicted gene shows all the characteristic elements of a complete gene and is closely flanked at both ends by other open reading frames which is a typical feature of herpesvirus gene arrangement⁷.

The average length of mature class I α chains consists of 345 (± 6) amino acids and the relative molecular mass (M_r) ranges from 39,000 (39K) to 48K, depending upon the variation in length and the number of carbohydrate side chains. The predicted length of the mature HCMV-H301 α chain is 350 amino acids. In contrast to only one to three glycosylation sites in HLA and H-2 class-I molecules, HCMV-H301 has 13 potential amino-linked glycosylation sites which makes an accurate prediction of the relative molecular mass impossible. The HCMV-H301 leader sequence (LD), consisting of 18 amino acids shows only little similarity but this is not surprising because it does not form part of the mature protein. Like other MHC class-I α chains, the extracellular region of the HCMV-H301 α chain can also be divided into the corresponding α -1, α -2 and α -3 domains. The α -1 domain contains no cysteine and shows a similarity of 21% to various α -1 domains as presented in Fig. 3. Details of how the similarities are calculated and an assess-

Fig. 3 (Opposite) alignment of the deduced HCMV-H301 amino-acid sequence to some α chains of class-I antigens from mouse (H-2), rat (RT1), human (HLA) and rabbit (RLA). Similar amino acids are marked by (:) for 50%-100% similarity and (.) for 25%-50% similarity. The four highly conserved cysteines (C) in the α -2 and α -3 domains are highlighted by asterisks (*). Extra cysteines are at positions 252, 269 and 275 for HCMV-H301, at position 71 for HLA-12.4 and at position 130 for H-2K^b, H-2K^k, H-2K^{w29.7}, H-2D^d. All potential amino-linked glycosylation sites (N-X-T/S) and the HCMV-H301 transmembrane region (TM) are underlined. Note, that the numbering used here does not correspond to the actual position of the amino acids in the native protein, because 'padding characters' (-) were introduced into the amino-acid sequences at various positions in order to maximize the similarity. The amino-acid sequences of the class-I α chains were taken from refs. 4, 12 and 20. The similarity with the MHC class-I antigens was found using the FASTP program of Lipman and Pearson²¹ searching the PseqIP protein-sequence data bank²⁰. No similarity was found to other herpes viruses (HSV 1, 2, VZV and EBV). The significance of the similarity was determined using the ALIGN program of Dayhoff *et al.*²² using a bias of 6 and a break penalty of 6. By random shuffling of the deduced HCMV-H301 protein sequence an assessment of the probability of the observed similarity occurring by chance was calculated on the basis of the probabilities of standardized scores for the normal distribution. A run of 60 permutations against the 80% consensus sequence of the class-I sequences presented above shows an alignment score of 13.58 standard deviation units (s.d.). The probability of this score occurring by chance is $< 10^{-20}$. In comparison, two other HCMV encoded membrane glycoproteins, gB²³ and gH (unpublished results), show alignment scores of 1.81 and 2.51 s.d. respectively. The probabilities of these scores are in both cases $> 10^{-3}$ indicating that they are 17 orders of magnitude more likely to occur by chance. The similarity numbers given in the text are calculated on the 80% consensus sequence (including padding characters) of all the class-I sequences presented. Similarities of less than 80% and amino-acid similarities are not included.

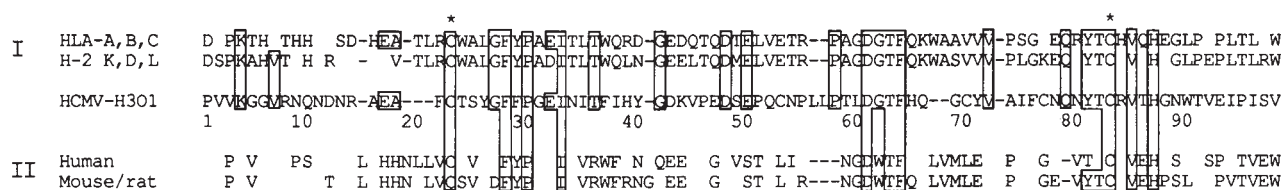


Fig. 4 Alignment of the deduced amino-acid sequence of HCMV-H301 α -3 domain with the consensus sequences of class-I α -3 chains (I) and class-II β -2 domains (II) from human (HLA-A, B, and C) and mouse (H-2K, D and L). The consensus sequences were taken from ref. 24. Boxes indicate sequence similarity of HCMV-H301 with class I and class II. Blank spaces indicate a consensus lower than 80% and 'padding characters' (-) were introduced to maximize similarity. The two conserved cysteines (C) are highlighted by asterisks (*).

ment of the significance of the overall similarity are given in the legend to Fig. 3. The α -2 domain shares the two conserved cysteines (position 110 and 184, Fig. 3) and shows a similarity of 20%. But the corresponding loop between the two cysteines consists of 72 rather than 62 amino acids. The α -3 domain shares the two highly conserved cysteines (position 223 and 281, Fig. 3) and the loop between the two cysteines contains exactly 55 amino acids. It contains three extra cysteines but some other class I molecules also have an extra cysteine in the α -1 or α -2 domain (see Fig. 3, legend). The similarity of 22% of the HCMV-H301 α -3 domain seems to be clustered mainly in three distinct regions (position 227-233, 260-263 and 277-283, Fig. 3). The α -3 domain of class I antigens is known to contain a binding site for β_2 -m. According to the crystal structure of HLA-A2 (ref. 8) this binding site is formed by a loop of six amino acids (position 258-263, Fig. 3). Four of the six residues are conserved in the viral α -3 domain, suggesting that HCMV-H301 also shares a functional β_2 -m binding site. To span the cellular membrane, the transmembrane sequence (TM) should be ~20 amino acids long and should consist of mainly uncharged, hydrophobic amino acids. The HCMV-H301 α chain has a potential TM sequence of 23 amino acids. The main feature of the class I cytoplasmic region (CY) are two potential phosphorylation sites S-D or E-X-S-L and K-G-G-X-Y (position 370 and 358, Fig. 3) at which cellular protein kinases can phosphorylate the serine (S)⁹ or the tyrosine (Y)¹⁰. The cytoplasmic region of HCMV-H301 contains no such potential phosphorylation site which could be of significance in the viral budding process.

Amino-acid sequence analyses have shown that β_2 microglobulin, class-I α -3 domains, class-II α -2 and β -2 domains and other members of the immunoglobulin superfamily¹¹ show a certain degree of similarity, suggesting that they all share a common ancestry^{4,6,11}. Comparison of the amino-acid sequence between the HCMV-H301 α -3 domain and the consensus sequences of class-I α -3 domains from human and mouse and of class II β -2 domains from human and mouse/rat, indicates that the origin of HCMV-H301 is presumably after class-I and class II genes diverged from their common precursor. The alignment of the HCMV-H301 α -3 domain with these consensus sequences clearly shows (1) that HCMV-H301 shares higher similarity with class I than with class II and (2) that all positions, where it shows similarity to at least one class II consensus sequence, are also shared with the class I consensus sequences. A comparison of the HCMV-H301 α chain to species-specific residues determined for human, mouse, rat and rabbit^{4,12} revealed no preferential similarity to either of these species. We conclude from this and the above evidence, that the predicted HCMV-H301 class I gene originated shortly after the class I and class II genes diverged and before the species-specific differentiation of the class I genes became apparent.

Evidence exists that cellular class I molecules can serve as receptors for adenovirus 2 (ref. 13), Semliki Forest virus¹⁴ and

HCMV^{3,5,15}. Based upon the findings, (1) that HCMV exists *in vivo* (in urine) as β_2 -m-coated particles¹⁵, (2) that cultured HCMV strain AD169 binds β_2 -m and competes for β_2 -m binding sites on human fibroblasts⁵, (3) that the addition of β_2 -m to the culture medium increases the amount of infectious, extracellular HCMV⁵ and (4) the identification of two β_2 -m binding HCMV envelope proteins $M_r = 36K$ and $65K$, respectively³, it has been proposed that the interaction of these two proteins with cellular class-I molecules via β_2 -m displacement or exchange might be a pathway for HCMV receptor-mediated infection⁵. The two proteins were specified as non-class I proteins because they did not coincide with the average relative molecular mass of 45K of class I antigens^{3,5}. According to our prediction HCMV-H301 could correspond to the 65K protein, assuming that seven or eight of the 13 potential glycosylation sites are actually glycosylated *in vivo*. Our discovery of a HCMV encoded class I-like membrane glycoprotein provides a probable explanation for the binding of β_2 -m by HCMV and supports the model proposed by Grundy *et al.*¹⁵. Once β_2 -m-coated HCMV comes in close contact with a target cell, exchange or displacement of HCMV-H301 bound β_2 -m with β_2 -m bound to cellular HLA class I antigens might take place and trigger internalization of the virus by receptor mediated endocytosis. The exact mechanism by which this might happen, however, is unclear and is in any case speculative and other pathways may exist.

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Loss of constitutional heterozygosity in colon carcinoma from patients with familial polyposis coli

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Recent studies have suggested a critical role of specific gene loss in several embryonic tumours¹⁻⁶ and certain adult cancers⁷⁻¹². In retinoblastoma, hemizyosity or homozygosity of a recessive mutant allele results in the loss of normal gene product²⁻⁴, and this seems to cause the manifestation of the disorder. Familial

polyposis coli (FPC) is a human autosomal dominant trait characterized by numerous adenomatous polyps of the colon and rectum, and a high incidence of colon carcinoma. Karyotype analyses have failed to detect specific deletion or translocation. We report the use of polymorphic DNA markers to look for the somatic loss of heterozygosity at specific loci. Investigation of 38 tumours from 25 FPC patients, and 20 sporadic colon carcinomas from 19 patients, revealed frequent occurrence of allele loss on chromosome 22, with some additional losses on chromosomes 5, 6, 12q and 15. The FPC gene-linked DNA probe C11p11 also detected frequent allele loss in both familial and sporadic colon carcinomas but not in benign adenomas. These results suggest the possible involvement of more than one chromosomal locus in the development of familial and sporadic colon carcinomas.

FPC is a genetic disorder transmitted as an autosomal dominant trait, and characterized by the development of numerous adenomatous polyps in the colon and rectum within the first few decades of life. The patients are at an extremely high risk of colorectal carcinoma if left untreated. General chromosomal instability¹³ and increased susceptibility to viruses^{14,15} and chemical carcinogens¹⁶ have been demonstrated in skin fibroblasts from patients with FPC. But little has been known about the mechanisms of tumorigenesis in FPC. Here we searched for somatic loss of particular chromosomal regions in colon carcinomas derived from patients with FPC, and

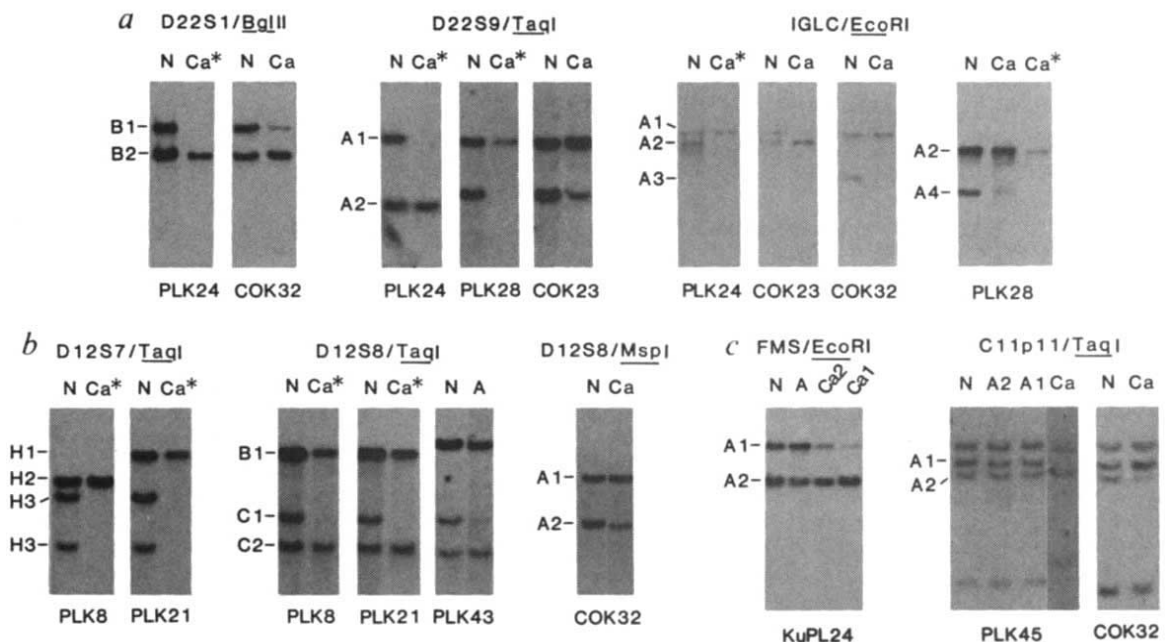


Fig. 1 Representative Southern hybridizations of chromosomes *a*, 22, *b*, 12q and *c*, 5, markers to normal tissue (N), adenomatous polyp (A), and colon carcinoma (Ca) DNA. Patient numbers were shown below the blots. PLK and KuPL indicate patients with FPC, and COK and KuCO indicate patients with sporadic colon carcinoma. Ca* indicates a tumour transplanted in a nude mouse. The letter and number on the left indicate an RFLP and polymorphic allele. The designation follows that of ref. 29 except for IGLC, where alleles are designated 1-4 according to decreasing length. *a*, The probe for D22S1 reveals an RFLP with fragments of 9.5 kilobases (kb) (B1) and 6.5 kb (B2) in *Bgl*II-digested DNA, and the probe for D22S9, with fragments of 5.8 kb (A1) and 3.2 kb (A2) in *Taq*I-digested DNA. The probe for IGLC detects a multi-allele RFLP with four fragments of 23 kb (A1), 18 kb (A2), 13 kb (A3) and 8 kb (A4) in *Eco*RI-digested DNA. *b*, The probe for D12S7 detects a *Taq*I polymorphism showing four alleles of 6.8 kb (H1), 5.1 kb (H2), 4.1 and 2.7 kb (H3), and 2.7 and 2.3 kb (H4, not observed). The probe for D12S8 reveals two RFLPs with fragments of 8.0 kb (B1) and 5.0 kb (B2, not observed), and 4.0 kb (C1) and 3.0 kb (C2) in *Taq*I-digested DNA, and an RFLP with fragments of 6.0 kb (A1) and 4.3 kb (A2) in *Msp*I-digested DNA. *c*, The probe for *c-fms* detects an *Eco*RI polymorphism with two fragments of 29 kb (A1) and 16 kb (A2), and the probe C11p11 detects a *Taq*I polymorphism producing two alleles of 4.4 kb (A1) and 3.9 kb (A2).

Methods. DNA with high relative molecular mass was isolated from surgical tumour specimens and/or their transplants into nude mice, and the corresponding normal tissues (skin fibroblasts and/or normal colon muscle) by the method of SDS-proteinase K and phenol/chloroform treatment, digested with restriction enzymes, electrophoresed in agarose gel, and transferred onto nitrocellulose membranes as described by Southern⁴¹. The membranes were pre-hybridized for 2 h at 41 °C in 50% formamide, 0.75 M NaCl, 2 mM EDTA, 0.1 M PIPES (pH 6.8), 1× Denhardt's solution, and 100 µg denatured salmon sperm DNA per ml. Hybridization was done for 20-24 h at 41 °C in the same solution as the pre-hybridization except for the addition of the radioactive probes. After hybridization, the membranes were washed three times in 50% formamide/2× SSC, then twice in 2× SSC/0.2% SDS, and exposed at -70 °C.

Table 1 Loss of heterozygosity in polyps and colon carcinomas from patients with FPC

Marker Enzyme Chromosome	L-myc <i>EcoRI</i> 1p32	CRYG <i>TaqI</i> 2q33-q35	D3S2 <i>MspI</i> 3p14-p21	D5S2 <i>MspI</i> 5pter-q35	<i>fms</i> <i>EcoRI</i> 5q34	C11p11 <i>TaqI</i> 5q21-q22	<i>myb</i> <i>EcoRI</i> 6q15-q24	COL1A2 <i>EcoRI</i> 7q21-q22	HRAS1 <i>BamHI</i> <i>MspI</i> 11p15	KRAS2 <i>TaqI</i> 12p12.1
PLK 8 Ca	1/2	1/2	—	—	1/2	—	—	1/2	1/2	—
PLK 12 Ca	—	1/2	—	—	—	—	—	1/2	—	—
PLK 15 A	—	1/2	1/2	—	—	—	—	—	—	—
PLK 21 Ca	—	1/2	—	—	—	—	—	1/2	—	—
PLK 24 Ca	—	1/2	1/2	—	—	—	1	1/2	—	1/2
PLK 25 A	1/2	1/2	1/2	—	1/2	—	—	—	—	—
PLK 27 Ca	—	1/2	—	—	—	—	—	—	1/2	1/2
PLK 27 A	—	1/2	—	—	—	—	—	—	1/2	1/2
PLK 28 Ca	1/2	1/2	1/2	—	—	—	1/2	1/2	1/2	—
PLK 28 A	1/2	—	—	—	—	—	1/2	1/2	1/2	—
PLK 35 Ca	1/2	—	1/2	—	—	—	—	—	1/2	—
PLK 35A	1/2	—	—	—	—	—	—	—	1/2	—
PLK 36 Ca	1/2	1/2	1/2	—	—	—	—	—	—	—
PLK 36 A	1/2	1/2	1/2	—	—	—	—	—	—	—
PLK 39 A	1/2	1/2	—	1/2	—	—	1/2	—	1/2	—
PLK 41 Ca	—	—	1/2	—	1/2	—	—	—	1/2	—
PLK 41 A	—	—	1/2	—	1/2	—	—	—	1/2	—
PLK 43 Ca	—	1/2	—	—	—	—	1/2	—	—	1/2
PLK 43 A1	—	1/2	—	—	—	—	2	—	—	1/2
PLK 43 A2	—	1/2	—	—	—	—	1/2	—	—	1/2
PLK 45 Ca	—	—	—	—	—	2	—	—	—	—
PLK 45 A1	—	—	—	—	—	1/2	—	—	—	—
PLK 45 A2	—	—	—	—	—	1/2	—	—	—	—
KuPL 8 A	—	1/2	—	1/2	1/2	—	—	1/2	—	1/2
KuPL 11 A	—	—	—	—	—	—	1/2	—	1/2	—
KuPL 15 Ca	—	—	—	—	—	—	1/2	—	1/2	—
KuPL 15 A	—	—	—	—	—	—	1/2	—	—	—
KuPL 18 A	1/2	1/2	—	—	—	—	—	1/2	1/2	—
KuPL 19 Ca	—	1/2	—	—	—	—	—	—	1/2	—
KuPL 19 A	—	1/2	—	—	—	—	—	—	1/2	—
KuPL 20 Ca	—	—	—	—	—	—	—	—	1/2	—
KuPL 22 A	—	—	—	—	—	—	—	—	1/2	—
KuPL 24 Ca1	—	—	—	—	2	—	—	1/2	—	—
KuPL 24 Ca2	—	—	—	—	2	—	—	1/2	—	—
KuPL 24 A	—	—	—	—	1/2	—	—	1/2	—	—
KuPL 25 A	1/2	1/2	—	—	1/2	—	1/2	—	1/2	—
KuPL 32 A	—	—	—	—	—	—	—	—	—	—
KuPL 33 A	1/2	1/2	—	—	—	—	—	1/2	1/2	—

continued opposite

examined whether the loss of allele is specific to FPC or is shared by the sporadic colon carcinomas. We also investigated whether or not such loss is detectable in the pre-cancerous polyps.

Twenty-five FPC patients (ten with only adenomatous polyps, five with colon carcinoma, and ten with both polyps and carcinoma) and 19 patients with sporadic colon carcinoma were examined to search for somatic loss of alleles at specific loci. DNA with high relative molecular mass was isolated from surgical specimens of adenomatous polyps, colon carcinomas, and corresponding normal colon muscle, mucosa or skin fibroblasts. In some cases, tumour specimens transplanted into nude mice were used as a source of tumour DNA. DNA was digested with appropriate restriction enzymes, and Southern hybridization was performed with several polymorphic DNA markers.

Loss of heterozygosity was observed in tumours (adenomas and carcinomas) from patients with FPC on five different chromosomes; 5, 6, 12, 15 and 22, with frequencies ranging from 21% to 29% (Table 1). Loss of heterozygosity was also observed in sporadic colon carcinomas on chromosomes 5, 6, 12 and 22, with frequencies ranging from 13% to 25% (Table 2). In chromosomes 1-3, 7, 11, 17 and 18, such loss was not detected in either FPC or sporadic colon carcinomas. Frequent occurrence of allele loss was seen on chromosome 22, using five polymorphic DNA markers (SIS, D22S1, D22S9, IGLC and IGLV). Out of 25 patients with FPC, 14 patients with carcinoma were informative for at least one of the five markers on chromosome 22. Of 14 informative cases, somatic loss of heterozygosity was observed in five carcinomas (36%), but not in adenomas from the same patients. Frequent allele loss on this chromosome was also observed in sporadic colon carcinomas (4/19 informative cases;

21%). These cases are displayed in Fig. 1a. Loss of heterozygosity was observed only in malignant colon carcinoma, and not in benign adenoma except for one case which had lost constitutional heterozygosity for chromosomes 6 and 12q. This suggests that the loss of heterozygosity observed here, is not responsible for the generation of benign adenomas, but is either the cause or the result of carcinogenesis.

Similar loss of heterozygosity was observed for the markers D12S7 and D12S8 mapped to 12q (Fig. 1b). Out of 19 informative patients with FPC, three colon carcinomas and one adenoma showed loss of heterozygosity for the markers (21%). Two of these four patients were also informative at the *KRAS2* locus on 12p, and these two tumours retained heterozygosity at this locus. This suggests that the loss of the allele on chromosome 12 is due to a partial deletion rather than the entire loss of the chromosome. As this loss was detected also in an adenoma (PLK43), this chromosomal region may play a role at the initial stage of carcinogenesis. The same loss was also observed in sporadic colon carcinomas (2/15 informative cases; 13%).

One patient with FPC showed loss of heterozygosity at the *c-fms* locus on chromosome 5 in two independent colon and rectal carcinomas, but not in adenoma (Fig. 1c). One of these carcinomas from this patient had also lost the heterozygosity for the *IGLV* locus on chromosome 22. Recently, the localization of the FPC gene on chromosome 5 (ref. 17) and allele loss on this chromosome in sporadic colon carcinomas¹⁸ have been reported. We used the probe C11p11, which is closely linked to the FPC gene, to examine whether or not the same loss should occur in the tumorigenesis in FPC. Out of 25 patients with FPC, only one was informative for this probe, and colon carcinoma from this patient PLK45 had lost the heterozy-

Table 1 continued

Marker Enzyme Chromosome	D12S7 <i>TaqI</i> 12q14-qter	D12S8 <i>TaqI</i> <i>MspI</i> 12q14-qter	D15S1 <i>MspI</i> 15q14-q21	D17S1 <i>MspI</i> 17p13-pter	D18S1 <i>TaqI</i> 18	sis <i>HindIII</i> 22q12-q13	D22S1 <i>BglII</i> 22q11-q13	D22S9 <i>TaqI</i> 22q11	IGLC <i>EcoRI</i> 22q11	IGLV <i>TaqI</i> <i>BamHI</i> 22q11
PLK 8 Ca	1	2	—	—	—	—	—	1/2	—	—
PLK 12 Ca	—	1/2	—	—	1/2	—	—	—	2	—
PLK 15 A	—	1/2	—	—	1/2	—	—	—	1/2	1/2
PLK 21 Ca	1	2	1	—	1/2	—	1/2	—	—	—
PLK 24 Ca	2	—	—	—	—	1	2	2	1	1
PLK 25 A	—	—	—	—	1/2	—	1/2	—	1/2	—
PLK 27 Ca	1/2	—	—	—	—	—	—	—	1/2	—
PLK 27 A	1/2	—	—	—	—	—	—	—	1/2	—
PLK 28 Ca	1/2	1/2	—	—	1/2	—	—	1	1	2
PLK 28 A	1/2	1/2	—	—	—	—	—	1/2	1/2	1/2
PLK 35 Ca	—	—	—	—	—	—	—	1/2	—	1/2
PLK 35 A	—	—	—	—	—	—	—	1/2	—	1/2
PLK 36 Ca	—	1/2	—	—	1/2	—	1/2	1/2	—	—
PLK 36 A	—	1/2	—	—	1/2	—	1/2	1/2	—	—
PLK 39 A	1/2	—	—	—	1/2	—	—	1/2	1/2	—
PLK 41 Ca	1/2	—	—	1/2	1/2	—	1/2	—	1/2	—
PLK 41 A	1/2	—	—	1/2	1/2	—	1/2	—	1/2	—
PLK 43 Ca	—	1/2	—	—	—	—	—	1/2	—	—
PLK 43 A1	—	2	—	—	—	—	—	1/2	—	—
PLK 43 A2	—	1/2	—	—	—	—	—	1/2	—	—
PLK 45 Ca	1/2	—	—	—	—	—	—	—	—	—
PLK 45 A1	1/2	—	—	—	—	—	—	—	—	—
PLK 45 A2	1/2	—	—	—	—	—	—	—	—	—
KuPL 8 A	1/2	—	1/2	—	—	—	—	—	—	1/2 1/2
KuPL 11 A	—	—	—	—	—	—	—	1/2	1/2	—
KuPL 15 Ca	1/2	—	—	2	—	—	—	2	—	2 1
KuPL 15 A	1/2	—	—	1/2	—	—	—	1/2	—	1/2 1/2
KuPL 18 A	—	—	—	—	1/2	—	—	—	—	1/2 1/2
KuPL 19 Ca	1/2	1/2	1/2	—	—	—	1/2	—	1/2	1/2 1/2
KuPL 19 A	1/2	1/2	1/2	—	—	—	1/2	—	1/2	1/2 1/2
KuPL 20 Ca	—	—	—	1/2	—	—	—	—	1/2	—
KuPL 22 A	—	—	—	1/2	—	—	—	—	—	1/2 1/2
KuPL 24 Cal	1/2	—	1/2	—	—	1/2	—	—	—	1/2 1/2
KuPL 24 Ca2	1/2	—	1/2	—	—	—	—	—	—	2 1
KuPL 24 A	1/2	—	1/2	—	—	1/2	—	—	—	1/2 1/2
KuPL 25 A	—	—	1/2	—	1/2	—	1/2	1/2	—	1/2 1/2
KuPL 32 A	1/2	1/2	1/2	—	—	1/2	—	1/2	1/2	—
KuPL 33 A	—	1/2	—	—	1/2	—	1/2	—	—	1/2 1/2

Tumour and normal DNA was digested with the restriction enzymes shown, fractionated by agarose gel electrophoresis, transferred to nitrocellulose or nylon membranes, and hybridized to probes for the indicated loci as described in Fig. 1. The probes used were number 6929 (*L-myc*)²⁸, p5G1 (CRYG)²⁹, pHF12-32 (D3S2)³⁰, pHF12-65 (D5S2)³⁰, the *PstI* fragment of *v-fms*³¹, C11p11 (ref. 17), 2.6 kilobase (kb) *EcoRI* fragment of *c-myc*³², NJ-3.5 (COL1A2)³³, the *HindIII*-*BamHI* fragment of *v-bas*³⁴ and VTR region of *c-Ha-ras*³⁵ (HRAS1), p640 (KRAS2)³⁶, pDL32B (D12S7)²⁹, p7G11 (D12S8)²⁹, pMS1-14 (D15S1)³⁰, pHF12-2 (D17S1)³⁰, pHF12-62 (D18S1)³⁰, the *PstI* fragment of *v-sis*³⁷, pMS3-18 (D22S1)³⁰, p22/34 (D22S9)³⁸, Hu-λ-C-R1-*HindIII* (IGLC)^{39,40} and pV3.3, a subclone from V4A (IGLV)²⁹. The phenotype observed in the tumour DNA is shown for every case where the normal DNA displayed heterozygosity. Heterozygosity is indicated by 1/2 (even though different pairs of alleles may be present for certain multi-allele markers). The continued presence of the larger allelic restriction fragment and loss of the smaller allelic fragment relative to normal tissue DNA is indicated by '1', and '2' indicates continued presence of the smaller allelic restriction fragment and loss of the larger fragment. Obscure case of allele loss is shown by (). Where the normal DNA was tested but did not display heterozygosity a dash is entered. The absence of an entry indicates that a marker was not tested or did not give a readable result for that particular patient. Ca, carcinoma; A, adenomatous polyps. The numbers after these abbreviations (for example Ca 1) indicate tumours developed at different sites of the colon in a particular patient.

gosity (Fig. 1c). The same loss was also observed in at least two sporadic colon carcinomas. Although the number of informative cases for this probe is limited, the frequency of allele loss seems to be very high, suggesting a key function in the development of colon carcinomas. There was no allele loss in the two adenomas from PLK45 where colon carcinoma had lost the heterozygosity. These two cases (KuPL24 and PLK45) suggest that the allele loss on chromosome 5 is involved in the development of carcinoma but not in polyp formation.

In total, eight FPC patients and eight sporadic cases were informative for at least one of the chromosome-5 markers, and two FPC cases and at least two sporadic cases have lost their heterozygosity for chromosome-5 markers (Tables 1 and 2). The frequency of allele loss seems to be relatively high and in accord with the results of Solomon *et al.*¹⁸.

Previous cytogenetic studies on colon carcinomas have shown several chromosomal abnormalities including loss of chromosomes 12p (ref. 19), 17p (ref. 20) and 18 (ref. 20). But our restriction fragment length polymorphism (RFLP) analyses using DNA probes p640, D17S1 and D18S1, mapped to 12p, 17p and 18, respectively, did not detect any chromosomal abnormality. This discrepancy may be due to the possible translocation of these chromosomal regions to other chromosomes, or to the

difficulties inherent to cytogenetic studies of complex karyotypes from solid tumour specimens.

In contrast to the previously reported tumours, somatic loss of alleles in the colon carcinomas is not specifically localized on any particular chromosome, although the nonrandomness is apparent from the fact that DNA markers for other chromosomes 1-3, 7, 11, 17 and 18 did not detect any chromosomal losses. Allele loss at the *c-Ha-ras* locus has been observed in several tumours, and its frequency has been reported to increase with tumour progression²¹. But we did not observe any qualitative or quantitative changes at this locus even in the DNAs extracted from tumours transplanted into nude mice (PLK 8, 21, 24 and 28). This suggests that the genome is remarkably stable, and therefore that the allele losses were not simply the result of chromosome instability in FPC and sporadic colon carcinomas.

Allele losses observed in this study could be interpreted in three ways: (1) An FPC gene may be located on one of the chromosomes where loss of heterozygosity was detected. (2) The allele losses may be secondary chromosomal events necessary for carcinogenesis, that follow the first event occurring at the FPC gene or other colon carcinoma-related gene(s) located on a different chromosome. (3) The allele losses may be the

Table 2 Loss of heterozygosity in sporadic colon carcinomas

Marker Enzyme Chromosome	L-myc <i>EcoRI</i> 1p32	CRYG <i>TaqI</i> 2q33-q35	D3S2 <i>MspI</i> 3p14-p21	D5S2 <i>MspI</i> 5pter-q35	fms <i>EcoRI</i> 5q34	C11p11 <i>TaqI</i> 5q21-q22	myb <i>EcoRI</i> 6q15-q24	COL1A2 <i>EcoRI</i> 7q21-q22	HRAS1 <i>BamHI</i> <i>MspI</i> 11p15	KRAS2 <i>TaqI</i> 12p12.1
COK 23 Ca	—	1/2	1/2	—	—	—	2	—	—	—
COK 26 Cal	—	—	—	—	—	—	—	—	1/2	—
COK 26 Ca2	—	—	—	—	—	—	—	—	1/2	—
COK 29 Ca	—	—	—	—	—	—	1/2	—	—	—
COK 29 A	—	—	—	—	—	—	—	—	—	—
COK 30 Ca	—	—	—	—	—	1/2	—	—	1/2	—
COK 31 Ca	—	1/2	1/2	1/2	—	—	1/2	—	1/2	—
COK 32 Ca	1/2	1/2	1/2	—	—	1	—	—	—	—
COK 34 Ca	1/2	1/2	—	—	—	—	1/2	1/2	—	—
KuCO 1 Ca	—	1/2	—	—	—	—	—	1/2	1/2	—
KuCO 2 Ca	1/2	1/2	1/2	—	—	—	—	1/2	—	—
KuCO 3 Ca	—	1/2	1/2	—	—	1	—	1/2	1/2	—
KuCO 4 Ca	1/2	1/2	—	—	—	—	1/2	—	1/2	1/2
KuCO 5 Ca	—	1/2	—	—	1/2	—	—	1/2	—	—
KuCO 13 Ca	—	—	—	—	1/2	—	—	1/2	1/2	1/2
KuCO 14 Ca	—	—	1/2	—	1/2	—	—	1/2	1/2	1/2
KuCO 15 Ca	—	—	—	—	—	—	—	1/2	—	1/2
KuCO 17 Ca	1/2	1/2	1/2	—	—	—	—	—	—	—
KuCO 18 Ca	1/2	1/2	1/2	—	—	(1)	—	—	—	—
KuCO 20 Ca	—	—	1/2	—	—	—	1/2	—	1/2	—
KuCO 21 Ca	1/2	1/2	1/2	—	—	—	—	1/2	—	—

Marker Enzyme Chromosome	D12S7 <i>TaqI</i> 12q14-qter	D12S8 <i>TaqI</i> <i>MspI</i> 12q14-qter	D15S1 <i>MspI</i> 15q14-q21	D17S1 <i>MspI</i> 17p13-pter	D18S1 <i>TaqI</i> 18	sis <i>HindIII</i> 22q12-q13	D22S1 <i>BglII</i> 22q11-q13	D22S9 <i>TaqI</i> 22q11	IGLC <i>EcoRI</i> 22q11	IGLV <i>TaqI</i> <i>BamHI</i> 22q11
COK 23 Ca	1/2	1/2	1/2	1/2	—	—	—	1	2	—
COK 26 Cal	—	—	—	—	1/2	—	—	—	1/2	—
COK 26 Ca2	—	—	—	—	1/2	—	—	—	1/2	—
COK 29 Ca	—	—	1/2	1/2	—	—	1/2	—	1/2	—
COK 29 A	—	—	1/2	1/2	—	—	—	—	1/2	—
COK 30 Ca	1/2	—	—	—	1/2	—	—	—	1/2	—
COK 31 Ca	1/2	—	1/2	1/2	—	—	—	—	1/2	—
COK 32 Ca	—	—	1	—	—	—	2	—	1	—
COK 34 Ca	—	—	1	—	1/2	—	1/2	1/2	—	—
KuCO 1 Ca	1/2	1/2	1/2	1/2	—	—	—	—	1/2	—
KuCO 2 Ca	—	—	—	—	—	—	—	2	2	—
KuCO 3 Ca	—	—	1/2	1/2	—	1/2	1/2	—	—	1/2
KuCO 4 Ca	—	—	—	—	1/2	—	—	1/2	1/2	1/2
KuCO 5 Ca	1/2	—	1/2	—	1/2	—	1/2	—	1/2	1/2
KuCO 13 Ca	1/2	1/2	1/2	—	—	—	—	1/2	1/2	—
KuCO 14 Ca	1/2	—	—	—	1/2	—	—	—	1/2	1/2
KuCO 15 Ca	1/2	—	—	—	—	—	2	—	1	1/2
KuCO 17 Ca	1/2	1/2	—	1/2	1/2	—	1/2	—	1/2	—
KuCO 18 Ca	—	—	—	1/2	—	—	1/2	—	—	—
KuCO 20 Ca	—	1/2	—	1/2	—	—	1/2	1/2	—	1/2
KuCO 21 Ca	1/2	—	—	—	—	—	1/2	1/2	1/2	1/2

For details refer to Table 1 legend.

result of abnormal mitotic events during tumorigenesis, and have no relation to the etiology of carcinoma. The FPC gene is now mapped to chromosome 5q (ref. 17), and therefore, allele loss on chromosome 5 may reflect the chromosomal events leading to the homozygosity or hemizygosity of the mutant FPC gene. Although the number of informative cases is small, high frequency of allele loss, especially that detected by the FPC gene-linked probe C11p11, suggests an important role of these chromosomal events in the development of colon carcinomas. No adenomas showed allele loss on chromosome 5 even in the cases of PLK45 and KuPL24 where their colon carcinomas had lost the heterozygosity for this chromosome. These data are consistent with the observations of Solomon *et al.*¹⁸, and suggest that the allele loss on chromosome 5 is involved in the carcinogenesis and not in the generation of benign adenomas.

Frequent occurrence of allele loss on chromosome 22 was seen in both familial and sporadic colon carcinomas. Loss of alleles on chromosome 22 has been shown in several tumours such as meningioma^{10,22}, acoustic neuroma⁸, bilateral acoustic neurofibromatosis⁹, von Recklinghausen neurofibromatosis²³ and Ewing's sarcoma²⁴. The human genome may contain a limited number of such loci whose defective alleles are predisposed to tumour formation in particular tissues, or gene loss at a specific locus/loci on chromosome 22 may promote tumour

cell growth. The allele loss on chromosome 22 observed here may be analogous to the case of MEN2, where allele loss is seen on chromosome 1 (ref. 25) although the gene is on chromosome 10 (refs 26 and 27). Our observation that allele loss occurs on different chromosomes including 5 and 22, suggests that the carcinogenesis in FPC is not simple but rather, multifactorial and complex. Evaluation of these chromosomal events, as well as the identification of the FPC gene on chromosome 5, will clarify the steps leading to the colon malignancy in FPC.

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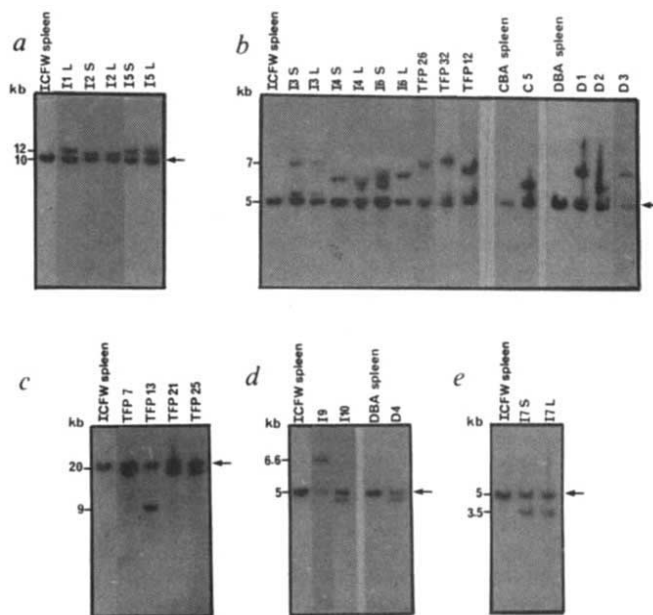


Fig. 1 Identification of a common region for SFFV proviral DNA integration in Friend tumours. Electrophoresis in a 0.8% agarose of 10 µg DNA digested with *Bam*HI (panels a, d and e), *Eco*RI (panel b) and *Eco*RV (panel c) from normal mouse spleen and from 22 Friend tumours, followed by Southern blot analysis. Filters were hybridized either to probe A (b and c), probe B (a), probe D (d) or to the probe E (e) described in Fig. 2a. Tumours numbered I1 to I10 were isolated from the spleen (IS) and from the liver (IL) of the same ICFW mouse infected with Friend viral complex (Lilly and Steeves SFFV strain). Spleen tumours TFP were isolated from ICFW mice infected with Friend viral complex (Ikawa SFFV strain). C5 and D1-D4 were spleen tumours isolated from CBA or DBA/2 mice infected with Lilly and Steeves SFFV strain. Arrows indicate germ-line bands (size in kb) detected in normal ICFW, CBA and DBA/2 spleen tissues in diploid amounts and in tumour tissues. The size of the additional bands differs among independent tumours. Of the six spleen and liver tumour pairs isolated from the same animal, five (I2, I3, I4, I5, I7) display identical extra bands. With the exception of I3S and I3L, all rearranged fragments appear to be in haploid amounts. In one additional tumour (I8) not shown here, the hybridization pattern was the same as for the normal DNA.

***Spi-1* is a putative oncogene in virally induced murine erythroleukaemias**

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Retroviral insertional mutagenesis has been proposed as an efficient mechanism to turn on or to increase the expression of oncogenes in several avian or mammal models. Integration site studies of avian leukosis virus, murine leukaemia and murine mammary tumour viruses led to the identification of highly conserved genes whose expression is induced or increased during leukaemogenesis, probably through enhancer elements present in the retroviral long terminal repeats¹⁻¹². This is reminiscent of the activation of cellular proto-oncogenes or putative oncogenes in numerous human tumours and leukaemias as a result of chromosomal translocations or DNA rearrangements. Here we report the characterization of a new putative oncogene isolated from a murine erythroleukaemia induced by the acute leukaemogenic retrovirus spleen focus forming virus (SFFV). An important and unusual feature of this genomic locus *Spi-1* (for SFFV proviral integration) is that rearrangements due to SFFV integration were found in 95% of the erythroid tumours studied. A 4.0-kilobase messenger RNA was detected in rearranged tumours. No *Spi-1* rearrangement was detected in other virally induced myeloid, lymphoid or erythroid tumours tested.

Even though no oncogene is present in its genome, the replication-defective murine type C SFFV induces an acute erythroleukaemia in mice which after an early proliferative step is followed by a clonal tumorigenic event¹³. The first step of SFFV-induced erythroleukaemia begins as early as 30 hours after *in vivo* viral injection. A non-clonal population of abnormal hyperbasophilic erythroid blast cells rapidly proliferates and differentiates *in vivo* (or *in vitro*) in the absence of erythropoietin, the physiological regulator of red-blood-cell production in mammals. Although these virus-infected cells give rise to a major splenomegaly (within a few days), their proliferative capacity is rather limited: they differentiate or die *in situ*, are not tumorigenic *in vivo*, and are not able to grow as permanent cell lines *in vitro*. Two to three weeks after viral infection, the first transplantable and immortalized erythroleukaemic cells can be detected^{14,15}. SFFV is crucial in both steps of this transformation process; injection of SFFV free of helper virus can reproduce the complete disease spectrum^{16,17}. The initial bursting out of erythropoietin-independent erythropoiesis probably results from the direct action of the SFFV genome product, the gp52-55 glycoprotein¹⁸. The mechanism by which SFFV induces the tumorigenic step, however, remains unclear. The DNAs extracted from these tumours do not transform NIH 3T3 cells and, with the exception of p-53 gene, no rearrangement or amplification of known oncogenes has been reported¹⁹.

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Fig. 2 *a*, Restriction map of the virus-cell junction fragment in tumour I5S cloned in the recombinant phage C45a1a1. The cloned DNA fragment was mapped by digestion of the phage insert with various combinations of restriction enzymes: *Bam*HI (B), *Eco*RI (E), *Eco*RV (E-V), *Hind*III (H), *Kpn*I (K), *Pst*I (P), *Pvu*II (Pv), *Sac*I (Sc), *Sal*I (S), *Xba*I (X). The size of each digestion product was calculated relative to a series of standard λ fragments. Probes A and B represent fragments of single-copy DNA localized in the cellular sequence adjacent to the 5' end of SFFV provirus. Probes are depicted in bold letters. SFFV viral-specific sequences are depicted as a thinner line and cellular DNA as a heavy line. An open box represents the SFFV 5' long terminal repeat (LTR). *b*, Map of the SFFV proviral integration sites in the *Spi-1* locus in the mouse genome. Data from restriction enzyme analysis of the unrearranged *Spi-1* locus cloned in phage E4e (thinner line), and of genomic DNAs from normal tissue and from tumour I5S, were used

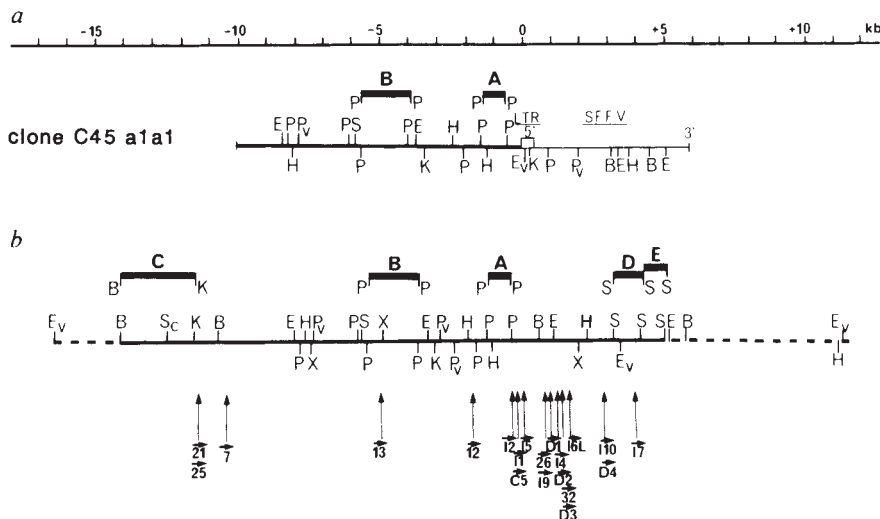
to establish a map of normal genomic locus *Spi-1*. Uncloned sequences were represented by dotted lines. Position zero is arbitrarily defined as the insertion site of the SFFV provirus in tumour I5S. Probes A, B, C, D and E are positioned by solid bars above the restriction map (probe A, *Pst*I-*Pst*I fragment from position -1.5 to -0.5; probe B, *Pst*I-*Pst*I fragment from position -5.3 to -3.6; probe C, *Bam*HI-*Kpn*I fragment from position -14 to -11.3; probe D, *Sal*I-*Sal*I fragment from position +3.2 to +4.2; probe E, a *Sal*I-*Sal*I fragment from position +4.2 to +5.0). Vertical arrows above tumour numbers designate the position of proviral integrations in the *Spi-1* region. Horizontal arrows indicate the direction of proviral integration 5' to 3'. Although consistent with the fragments resulting from *Eco*RI, *Eco*RV, *Bam*HI, *Hind*III digests of each tumour DNA, the orientation of the proviruses in tumour DNAs have not been verified by hybridization to viral probes, because of lack of specific SFFV LTR and *gag* probes. The maps deduced from this restriction fragment analysis are compatible with the presence of an integrated full-size SFFV genome, except in the case of D3, in which a provirus presented a deletion (1.5 kb) in the *gag-pol* region. **Methods.** DNA from the ICFW spleen tumour I5S was partially digested with *Mbo*I. Fragments ranging in size from 15–20 kb were cloned into the *Bam*HI site of the bacteriophage EMBL3 (ref. 23). The bacteriophage library (10^6 clones) was screened for positive phage plaques with the SFFV *env* probe: fragment *Bam*HI-*Sma*I in the 5' *env* gene²⁰. Fifty positive recombinant phages were identified and isolated by at least three cycles of plaque purification. Phage DNA stocks were prepared and subsequently tested for their ability to hybridize with the synthetic oligonucleotide probe specific for SFFV *env* gene²¹. The 20 resulting positive phages were mapped by digestion with various restriction endonucleases and SFFV-specific fragments were identified by blot hybridization using *env*, LTR and oligonucleotide SFFV probes. Phage inserts were subcloned in p-Gem-3 vector and tested for the presence of repetitive sequences by hybridization on normal mouse DNA. Phage C45a1a1 gave rise to probes A and B which revealed genomic rearrangements in various tumour DNAs, irrespective of the mouse and the SFFV viral strains used. Probe A was subsequently used to screen 5×10^5 recombinant clones from the same library. Three recombinant phages isolated from positively reacting plaques were purified and analysed by restriction mapping and hybridization with SFFV probes. Phage E4A insert that contained no viral fragments corresponded to the normal allele of the *Spi-1* genomic locus. Three probes (C, D and E) representative of unique cellular sequences were selected and subcloned into the plasmid vector p-Gem-3.

Earlier studies using SFFV-specific probes^{20,21} have shown that extra SFFV proviral bands could be detected in the genome of all the Friend tumour cells analysed^{21,22}. To search for a putative oncogene which would correspond to common SFFV integration sites, we constructed a genomic library from a DNA which was prepared from a tumour (I5S) harbouring five additional proviruses and partially digested by *Mbo*I. The molecular cloning of virus-host junction fragments was achieved using purified EMBL-3 arms. After ligation and *in vitro* packaging, 10^6 recombinant phages were screened successively with a SFFV-*env* probe and with a synthetic oligonucleotide probe specific for SFFV (ref. 21). Twenty positive phages were analysed in detail to demonstrate the presence of the SFFV genome. Ten phages were selected on the basis of restriction mapping analysis. Each insert represented a junction fragment that extended from a viral fragment, showing the characteristic structure of the SFFV genome²⁴ into the flanking cellular DNA. Phage inserts were subcloned into a p-Gem-3 vector to select for fragments free of repetitive sequences. From these 10 phages, we derived DNA fragments representative of flanking cellular sequences, and these fragments were used to probe genomic DNA prepared from the original tumour I5S; four of the five SFFV integration sites detected in this tumour genome were thus identified. These probes were then used to look for DNA rearrangements in other SFFV-induced tumours isolated *in vivo*; three out of four integration sites appeared to be unique, and the fourth was represented in the phage C45a1a1.

Two fragments were isolated from this phage C45a1a1 (Fig.

2a). Probe A (a 1.0-kilobase *Pst*I-*Pst*I fragment) is localized 500 base pairs (bp) upstream from the 5' SFFV LTR and probe B (1.7-kilobase *Pst*I-*Pst*I fragment) is 5 kilobase (kb) away from the virus on the same side relative to probe A. Both probes were used to look for rearrangements in genomic DNA prepared from tumours induced by various SFFV viral strains (Lilly-Steeves strain, Ikawa strain) in different mouse strains (ICFW, DBA2 and CBA) (see legend to Fig. 1). The genomic DNAs were digested with a set of four restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Eco*RV). The use of these enzymes with either probe permitted visualization of DNA fragments spanning over 20 kb of chromosomal DNA (Fig. 1). Out of a panel of 22 tumours, 17 tumours displayed a rearranged allele.

To analyse further this common region of SFFV proviruses integration in erythroblastic tumours (*Spi-1*), we looked for additional probes by screening 5×10^5 recombinant phages from the I5S-tumour-DNA phage library with probe A. Three positive clones were obtained. One clone (phage E4a) correspond to the germ-line allele of the *Spi-1* genomic locus spanning a region of about 20 kb of cellular DNA. A normal restriction map of the *Spi-1* allele was thus established by analysis of the cloned DNA and the genomic DNA (Fig. 2b). Three single-copy probes (probe C, a 2.7-kb *Bam*HI-*Kpn*I fragment; probe D, a 1.0-kb *Sal*I-*Sal*I fragment; probe E, a 0.8-kb *Sal*I-*Sal*I fragment) were derived by subcloning fragments from E4a phage clone into p-Gem-3 vector. Using DNAs digested with *Eco*RI, *Eco*RV or *Bam*HI, these probes revealed rearrangements in four other tumours (Fig. 1). In all cases the germ-line allele was present,



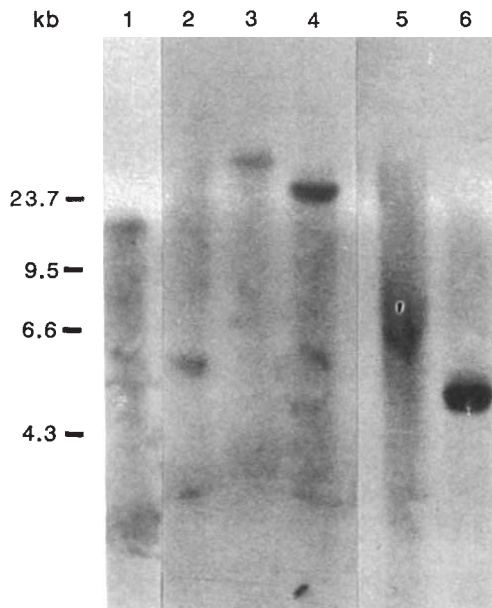


Fig. 3 Sequences homologous to *Spi-1* are present in various vertebrates. Cellular DNAs (10 µg) from the indicated sources were digested with *EcoRI*, electrophoresed in 0.8% agarose, transferred to nitrocellulose and annealed with probe A in 50% formamide, $5 \times \text{SSC}$ at 40 °C. After washing in $0.5 \times \text{SSC}$ at 45 °C, the filter was exposed to X-ray film for 3 d. Lane 1, mink kidney cells; lane 2, dog thymus cells; lane 3, chicken erythrocytes; lane 4, rat kidney cells; lane 5, human peripheral blood cells; lane 6, mouse spleen cells. Numbers on the left (in kb) refer to the positions of fragments of *HindIII*-digested λ phage.

and no amplification was observed. When a rearranged gene was detected in the DNA of a spleen-derived tumour (IS), the same rearrangement was observed using DNA of liver-derived tumour (IL) isolated from the same infected animal, except in one case (out of 6), where additional bands were observed. This could result from the presence in the spleen of a mixture of two populations of tumorigenic cells. This finding of similar rearrangements in independently isolated tumours derived from the same animal supports the hypothesis that these rearrangements were already present in the original population of tumour cells, and consequently could be an initial event during the malignant step.

The additional fragments observed in SFFV-induced tumours were shown to result from SFFV integration by Southern blot analysis. The nature of the virus, the precise location of the viral integration site, and the orientation of the virus relative to the cellular DNA, were deduced after digestion with different restriction endonucleases and hybridization with the probes A, B, C, D and E. In Fig. 2b the position and orientation of the SFFV proviruses in the different tumours are indicated. All rearrangements were due to SFFV proviral integration. Figure 2b shows that all proviruses were in the same orientation and most of them clustered in a region spanning 3 kb.

To determine whether this common integration site was specific for SFFV and for acute murine erythroleukaemias, we analysed DNA prepared from 4 lymphoid-, 11 myeloid- and 14 erythro-leukaemias induced by the helper virus of the Friend complex. No rearrangements were observed, suggesting that this site is specific for SFFV-induced erythroleukaemias (data not shown). We then investigated whether this site could be a known oncogene, or one of the integration sites previously described in various viral-induced leukaemias or tumours. The phages C45a1a1 and E4a were hybridized with oncogene-specific probes

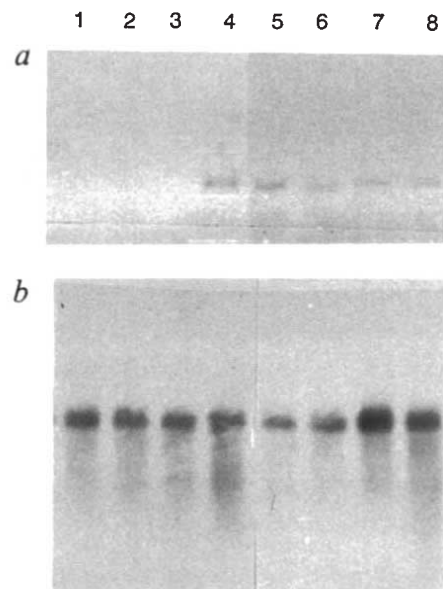


Fig. 4 Northern blot analysis of Friend erythroleukaemia poly (A⁺) RNAs. RNAs were prepared as previously described²⁶ and poly(A⁺) cytoplasmic RNAs were selected on oligo(dT) columns. RNAs (5 µg) were denatured with glyoxal, separated on 1% agarose gels, transferred to nitrocellulose and hybridized successively with the various ³²P-labelled nick-translated *Spi-1* probes (specific activity 2.5×10^8 c.p.m. per µg) for 24 h with 10^6 c.p.m. ml⁻¹. The filters were washed twice in $0.1 \times \text{SSC}$ 0.1% SDS at 60 °C for 30 min. *a*, Hybridization with probe C of the *Spi-1* region described in Fig. 2. Lane 1, normal spleen cells; lane 2, spleen cells of phenylhydrazine treated control mouse; lane 3, hyperbasophilic Friend cells from spleen of mice infected 10 d earlier with the Friend viral complex; lanes 4, 5, 6, 7, 8, five Friend tumour cells (D3, D2, C5, I2S, and I6S respectively) described in Fig. 1 legend. *b*, Filters subsequently hybridized with the rat glyceraldehyde-3' phosphodehydrogenase probe to check for uniform loading.

(*K-ras*, *H-ras*, *N-ras*, *erb-A*, *erb-B*, *rel*, *sis*, *mos*, *myb*, *myc*, *fms*, *fos*) and putative oncogene-specific probes (*int-1*, *int-2*, *pvt-1*, *Pim-1*, *Fim-1*, *Fim-2*, *Fis*). We also used these probes to look for rearrangements in the tumour cellular DNAs; this proved to be negative, and agrees with the lack of amplification and genomic rearrangement of these oncogenes and putative oncogenes in these Friend tumours (our unpublished data). *Spi-1* is different from the p53 oncogene, which is rearranged in certain SFFV-induced tumours^{19,25}, as well as in certain SFFV-induced tumours we have analysed (unpublished data), and also from the *erb-A*, *erb-B* and *H-ras* oncogenes which induce avian and murine erythroleukaemias.

The evolutionary conservation of the *Spi-1* locus was examined in cellular DNAs from different vertebrate species. These DNAs were digested with *EcoRI* and blot-hybridized with probe A under conditions of moderately reduced stringency. As illustrated in Fig. 3, *EcoRI* fragments that hybridized with probe A were present in DNA digests from birds and several mammals, including man, demonstrating that this DNA fragment is well-conserved throughout the evolution of vertebrates.

To examine whether insertion of a SFFV provirus in the *Spi-1* region was associated with the presence of polyadenylated RNAs, we looked for RNA expression with various *Spi-1*-derived probes. Probe C, located 10 kb away from the viral integration cluster (see Fig. 2), detected a 4.0-kb RNA transcript in SFFV-induced tumours (Fig. 4), but not in normal tissues or

in non-tumorigenic hyperbasophilic Friend cells. The size of this RNA was similar to that of the *myb* and *fms* transcripts. But *Spi-1* appeared to be clearly distinct from these oncogenes, as the pattern of expression of *myb*, *fms* and *Spi-1* in these tumours was quite different (data not shown).

Thus *Spi-1* contains sequences that represent single-copy elements in the mouse genome. These sequences are conserved among vertebrate species and are involved in 95% of SFFV-induced erythroid transplantable tumours, but not in other haemopoietic malignant tissues; this SFFV integration is associated with the expression of a 4.0 kb mRNA. The high frequency

of *Spi-1* involvement in SFFV-induced tumours strongly indicates that it corresponds to an early and essential step in the tumorigenesis in the Friend disease.

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Characterization of ribosomal frameshifting in HIV-1 *gag-pol* expression

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Based on precedents from other retroviruses¹, the precursor of the human immunodeficiency virus (HIV-1) reverse transcriptase is predicted to be a polyprotein with a relative molecular mass (M_r) of 160,000 (160K) encoded by both the viral *pol* gene and the upstream *gag* gene. These two genes lie in different translational reading frames, with the 3' end of *gag* overlapping the 5' end of *pol* by 205 or 241 nucleotides²⁻⁴. Thus, production of the *gag-pol* fusion protein would require either messenger RNA processing or translational frameshifting. The latter mechanism has been shown in the synthesis of the *gag-pol* proteins of two other retroviruses, Rous sarcoma virus (RSV)⁵ and mouse mammary tumour virus (MMTV)^{6,7}. Here we report that translation of HIV-1 RNA synthesized *in vitro* by SP6 RNA polymerase yields significant amounts of a *gag-pol* fusion protein, indicating that efficient ribosomal frameshifting also occurs within the HIV-1 *gag-pol* overlap region. Site-directed mutagenesis and amino-acid sequencing localized the site of frameshifting to a UUA leucine codon near the 5' end of the overlap.

Many, and probably all, retroviruses synthesize *gag-pol* fusion proteins, which are later cleaved by a virus-encoded protease to yield the mature *pol* proteins responsible for reverse transcription and integration¹. The genetic structure of the *gag-pol* domains of retroviral genomes apparently precludes synthesis

of this fusion protein, however, as *gag* and *pol* are either separated by an in-frame termination codon, overlapping in different reading frames, or are interrupted by a third gene (encoding the protease) which overlaps them both¹. To circumvent these apparent blocks to synthesis of *gag-pol* fusion proteins, the four retroviruses so far examined use three different strategies: stop-codon readthrough in the case of murine leukaemia virus (MLV)⁸ and feline leukaemia virus (FeLV)⁹, and single and double ribosomal frameshifting in RSV⁵ and MMTV^{6,7} respectively.

The *gag-pol* domain of HIV-1 resembles most closely that of RSV in that the two genes overlap directly (with *pol* in the -1 frame with reference to *gag*), although the *gag-pol* overlap is considerably larger in HIV-1 (205 or 241 nucleotides as compared to 58 nucleotides for RSV)^{2-4,10} (Fig. 1a). This region of the HIV-1 genome is apparently represented in a single mRNA species, the genome-length RNA, which is presumed to encode two proteins: the *gag* precursor, Pr55 *gag*, and a *gag-pol* fusion (160K)¹¹. Typically, retroviral *gag* and *gag-pol* precursor proteins are synthesized at a ratio of about 10-20:1 (ref. 1).

To test whether the HIV-1 *gag-pol* fusion protein is also produced by ribosomal frameshifting, we used an experimental protocol that had previously allowed us to show frameshifting during the expression of the RSV and MMTV *pol* genes^{5,6}. A DNA clone of HIV-1 (strain SF-2 (ref. 2)) encompassing the complete *gag-pol* domain was inserted downstream of the SP6 promoter¹² to form the plasmid pAGP (Fig. 1a). Linearization of pAGP at an *NdeI* site downstream of *pol* followed by transcription by SP6 RNA polymerase yields a unique species of RNA (N1-RNA) in which *gag* and *pol* are in their genomic, out-of-frame configuration. Translation of N1-RNA in a rabbit reticulocyte lysate system will, by normal translation, produce the *gag* protein, Pr55^{gag}, if some fraction of ribosomes shift into the -1 frame during translation of the 205 nucleotide (nt) *gag-pol* overlap, the 160K *gag-pol* fusion protein (referred to here as Pr160^{gag-pol}) will also be produced.

As shown in Fig. 1b, the unprecipitated translation products of N1-RNA include proteins of the correct molecular mass for Pr55^{gag} and Pr160^{gag-pol} (lane 1). Both of these proteins can be precipitated by an antiserum against the HIV-1 *gag* protein (lane 2); as expected, only the larger is recognized by an antiserum

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Fig. 1 Ribosomal frameshifting *in vitro*. *a*, The plasmid pAGP was constructed by inserting a 5.0 kilobase (kb) fragment from a DNA clone of HIV-1 (strain SF-2) extending from a *Sac*I site upstream of the *gag* initiator (position 225 in ref. 2) to an *Eco*RI site downstream of *pol* (position 5,296 in ref. 2) into the plasmid pSP64 (ref. 12) that had previously been digested with *Sac*I and *Eco*RI. The first 19 and last 16 nt of the 205 nt *gag-pol* overlap are shown translated in both the *gag* (above) and *pol* (below) frames. Linearization of pAGP with either *Eco*RV or *Nde*I, followed by *in vitro* transcription with SP6 RNA polymerase, yields the two RNAs shown (RV-RNA and N1-RNA). *b*, Translation of N1- and RV-RNA. The 35 S-labelled *in vitro* translation products of N1- and RV-RNA were electrophoresed through a 10% SDS-polyacrylamide gel either without immunoprecipitation (lanes 1 and 5) or after precipitation with anti-*gag* (lanes 2 and 6), anti-*pol* (lanes 3 and 7) or non immune rabbit serum (lanes 4 and 8). The gel was soaked in AMPLIFY (Amersham) and the proteins visualized by fluorography. The expected sizes of the *gag* and *gag-pol* proteins and positions of relative molecular mass standards (K) are shown. *In vitro* transcription, rabbit reticulocyte translations and immunoprecipitation reactions were carried out as described⁵, except that the translation reactions were stopped after 15 min due to the instability of the *gag-pol* proteins. The anti-*gag* and anti-*pol* antisera were raised against purified HIV-1 proteins produced in *Escherichia coli*. The *gag* antigen corresponds to p25^{gag}, the central *gag* protein¹⁷; the *pol* antigen is analogous to p31^{pol}, the presumed integrase, encoded at the 3' end of *pol*¹⁸.

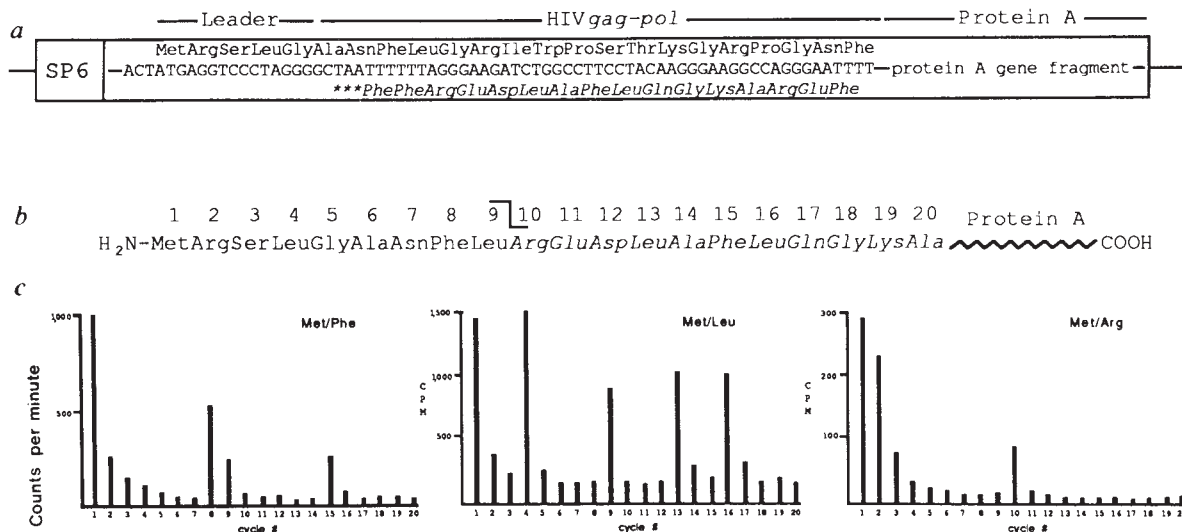
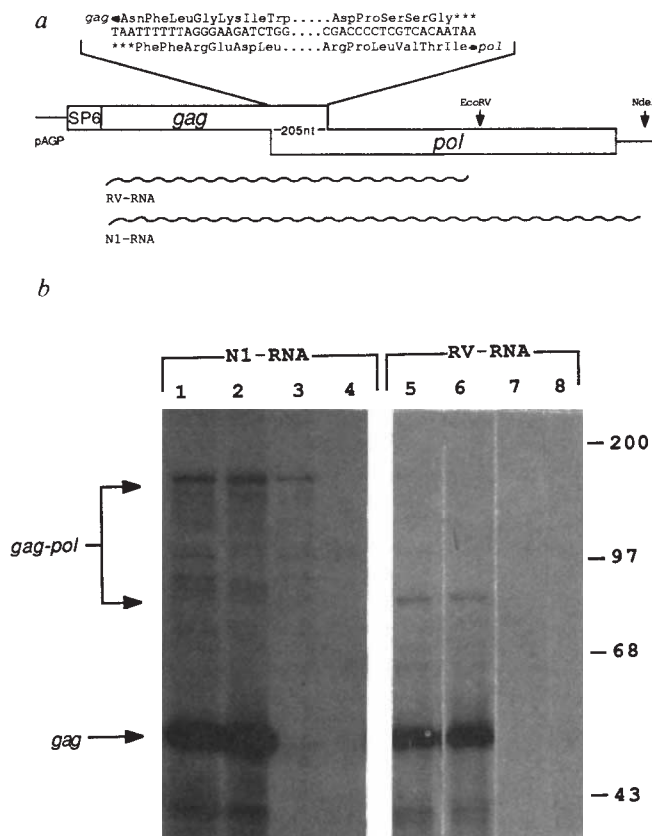


Fig. 2 The amino-acid sequence at the HIV-1 frameshift site. *a*, The plasmid pHSS was constructed by ligating a double-stranded oligonucleotide containing the first 50 nt of the HIV-1 overlap into an SP6 vector¹² between a short leader sequence (an initiator methionine codon plus five additional codons) and a portion of the *Staphylococcus* A-protein A gene (extending from position 742 to 2,001 in ref. 19). (The A-gene-containing vector pRIT2T was purchased from Pharmacia.) The A-gene segment is continuous with the *pol* frame of the HIV-1 insert. The leader sequence, which is in the *gag* frame, includes the first four amino acids of the chick pre-lysozyme protein²⁰, one of a small number of proteins known not to be amino-terminally acetylated during rabbit-reticulocyte lysate translation²¹. The two open reading frames are translated through the HIV-1 insert (*gag* above the nucleotide sequence, *pol* below it and in italics). *b*, The predicted amino-terminal amino-acid sequence of the fusion protein produced upon translation of pHSS-directed RNA, based on the model of frameshifting presented in the text. The predicted point of transition from the *gag* to *pol* frames is indicated (7); the *pol*-encoded amino acids are shown in italics. *c*, Radioactivity profiles of automated Edman degradation of pHSS-encoded protein synthesized *in vitro* in the presence of [35 S]methionine and either [3 H]phenylalanine (left), [3 H]leucine (centre) or [3 H]arginine (right). 500 μ l rabbit-reticulocyte translations were performed as described⁵, except that 25 μ g ml $^{-1}$ α_2 -macroglobulin (Boehringer) was added to inhibit proteolysis. The resulting fusion protein was purified using rabbit IgG-Sepharose (Pharmacia)²². The purified protein was subjected to 20 cycles of Edman degradation on an Applied Biosystems model 470A gas-phase protein sequencer using standard 03CATZ cycles; the products of each cycle were dried under vacuum, resuspended in scintillation fluid and counted in the 3 H channel.

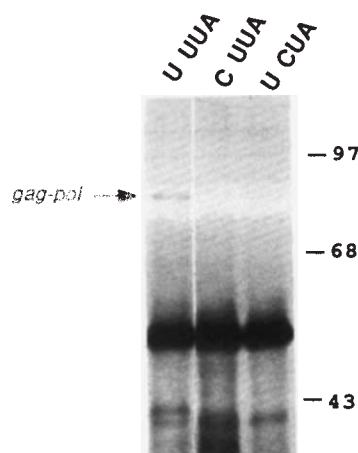


Fig. 3 Two point mutations in the HIV-1 U UUA sequence strongly inhibit frameshifting. A single-stranded DNA vector (M13) containing an HIV-1 *gag-pol* insert was used as the template for site-directed mutagenesis²³. Each of the first two thymidine residues in the overlap sequence T TTA (see Fig. 1a) were separately converted to cytosines, using 22-residue synthetic oligonucleotides containing the desired mutations. Double-stranded DNA fragments encompassing these mutations were isolated from the replicative form of the mutant vectors and used to replace the analogous fragment in the plasmid pAGP for *in vitro* analysis. The presence of the mutations were verified by DNA sequencing²⁴. Translation of wild-type and mutant RV-RNAs (Fig. 1a) was followed by immunoprecipitation with anti-p25^{gag} antiserum (see legend to Fig. 1) and separation of the products on a 10% SDS-polyacrylamide gel. The expected size of the gag and gag-pol proteins and positions of relative molecular mass standards (K) are indicated.

directed against pol (lane 3). The other minor, high molecular-mass protein species observed after N1-RNA translation (Fig. 1b) probably result from premature transcriptional or translational termination or internal translational initiation within *pol*.

To measure more accurately the ratio of the gag to gag-pol proteins and thereby estimate the frameshifting efficiency, we translated a shorter HIV-1 gag-pol RNA, RV-RNA (synthesized from *EcoRV*-linearized pAGP; Fig. 1a). RV-RNA also yields Pr55^{gag} as well as the predicted gag-pol fusion of 80K (Fig. 1b, lane 5). The two proteins are precipitable with the anti-gag serum (lane 6). But because the domain recognized by our anti-pol serum is encoded near the 3' end of the gene, the shorter gag-pol fusion is not recognized by this serum (lane 7). The ratio of the two proteins (as determined from the amount of radioactivity in the excised gel slices after correction for differential methionine content) is about 8:1, indicating a frameshifting efficiency of 11%. This efficiency is higher than the 5% we observed within the RSV *gag-pol* overlap⁵, although not as high as was measured for the upstream (*gag-x/pro*) overlap in MMTV, where about one in four ribosomes changes frame⁶.

To identify the site at which frameshifting occurs within the HIV-1 *gag-pol* overlap, we first noted the sequence U UUA (where the UUA is a codon in the *gag* frame) near the 5' end of the overlap (Fig. 1). This sequence also appears in the *gag-pol* overlap of RSV⁵ and the *pro-pol* overlap of MMTV⁶. We have previously proposed⁶ that -1 frameshifting at this sequence might be mediated by slippage of the leucyl transfer RNA reading the UUA codon back to the -1-frame UUU codon. (Amino-acid sequencing and site-directed mutagenesis is consistent with this model for RSV (T.J., F.R.M. and H.E.V., in preparation).) To test this model with HIV-1, we first determined a portion of the amino-acid sequence of a protein produced *in vitro* through frameshifting on an HIV-1 RNA. We cloned a

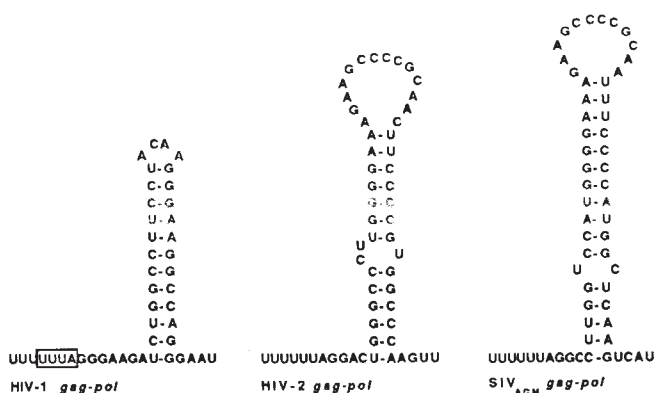


Fig. 4 Comparison of the HIV-1 frameshift site and potential stem-loop structure with homologous regions from the HIV-2 (ref. 13) and SIV_{AGM} (ref. 14). The HIV-1 frameshift site is shown boxed.

synthetic DNA fragment corresponding to the first 50 nt of the HIV-1 overlap into an SP6 vector designed to allow rapid purification and sequencing of *in vitro*-synthesized proteins, producing the plasmid pHSS (Fig. 2a). The mechanism of frameshifting proposed above would result in a pHSS-encoded fusion protein with the amino-acid sequence Phe-Leu-Arg at positions 8-10 (Fig. 2b), where the arginine is the first amino acid encoded in the *pol* frame.

The histograms shown in Fig. 2c record the amount of radioactivity in the first 20 cycles of Edman degradation performed on purified, differentially labelled fusion protein. The peaks of radioactivity at positions 8, 9 and 10 in the phenylalanine-, leucine- and arginine-labelled samples, respectively, confirm that the shift into the *pol* frame occurs primarily after the decoding of the leucine UUA codon at position 9 and are consistent with the model for frameshifting presented above. The other peaks, including that at position 1, are also predicted by the methionine, phenylalanine, leucine and arginine codons, either upstream of the frameshift site in the *gag* frame or downstream of this site in the *pol* frame (Fig. 2b). It appears, however, that ~30% of frameshifting ribosomes do not insert leucine at position 9, based on the amount of radioactive leucine at this position and that expected given the amounts at positions 4 and 13 (Fig. 2c, middle). Those ribosomes that do not insert leucine at position 9 may incorporate phenylalanine, as there is a small peak at this position in the [³H]phenylalanine label (Fig. 2c, left) that cannot be completely accounted for by carry-over of the [³H]phenylalanine at position 8 (Fig. 2b). Perhaps after slipping from the UUA codon and before peptidyl transfer and translocation, a small fraction of the leucyl tRNA is released from the A site, and the exposed A-site UUU triplet then decoded by a phenylalanyl tRNA.

Although the amino-acid sequence at the transition from the *gag* to *pol* frames is consistent with the slippage model, other mechanisms, for example two-nucleotide translocation by the tRNA reading the last 0-frame codon (UUA), would produce the same amino-acid sequence. To test more directly whether leucyl tRNA slips back to the -1 frame, we constructed two site-directed mutations, converting each of the first two uracyl residues of the sequence U UUA to cytosines. We would expect these mutations to inhibit frameshifting by disrupting the site to which the UUA-reading tRNA slips (in the C UUA mutant) or by changing the UUA codon itself and thereby specifying a tRNA with an anticodon that is less likely to pair in the -1 frame (the U CUA mutant). As is shown in Fig. 3, both of these mutations strongly inhibit frameshifting *in vitro*, supporting the slippage model. Also, the absence of gag-pol expression in these two mutants suggests that frameshifting in the HIV-1 *gag-pol* overlap is confined to this single site near the 5' end.

The positioning of the HIV-1 frameshift site results in translation of both *gag* and *pol* sequences in the *gag-pol* overlap. The viral protease is encoded in the *pol* frame beginning 55 amino-acids downstream of the frameshift site (S. Oroszlan and S. Venkatesan, personal communications); the function of the *pol*-encoded amino acids between the frameshift site and the protease domain is unclear.

The amino-acid sequence and site-directed mutagenesis implicate the U UUA sequence as necessary for frameshifting within the HIV-1 overlap. We do not, however, believe that this sequence is sufficient to cause such a high degree of ribosomal shifting. More extensive mutational analysis of RSV has shown the importance of nucleotides just 5' to this virus's U UUA sequence (T.J., F.R.M. and H.E.V., in preparation) and we have previously proposed that stem-loop structures situated just 3' to the frameshift sites might be important in the retroviral frameshifting mechanism⁶. (Deletion and site-directed mutational analysis of the RSV stem-loop supports this proposal (T.J., H. Madhani and H.E.V., in preparation).) The HIV-1 frameshift site is followed closely by a G-C rich stem-loop structure (Fig. 4). Two other retroviruses, HIV-2 (ref. 13), and the simian immunodeficiency virus (SIV) (ref. 14, 15) also contain within their *gag-pol* overlaps U UUA sequences followed by G-C rich stem-loop structures (Fig. 4). The U UUA sequences are included in regions of identity with HIV-1 that are 9 nt in length, but the sequence composition of the stem-loop structures are very different. We expect that these other viruses also use ribosomal frameshifting at this site to produce their *gag-pol* fusion proteins. A complex set of transcriptional and post-transcriptional mechanisms has been proposed to regulate HIV-1 genes¹⁶.

As has been suggested for some of the other mechanisms, a specific inhibitor of the frameshifting process could be an effective means of blocking HIV-1 replication.

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A pseudoknotted RNA oligonucleotide

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The diverse functions of RNA, which include enzymatic activities¹, regulatory roles in transcription² and translation³, are made possible by tertiary structure. Computer algorithms^{4,5} can predict the secondary structure of an RNA molecule using free-energy parameters for base pairing and stacking, loops and bulges^{6,7}. However, with the exception of transfer RNA, little is known about the structures and thermodynamics of interactions involved in the tertiary structure of RNA. Recently, it has been proposed that a novel form of RNA folding called pseudoknotting occurs at the 3' end of certain viral RNAs from plants⁸. A pseudoknot involves intramolecular pairing of bases in a hairpin loop with a few bases outside the stem of the loop to form an additional stem and loop region (Fig. 1). If each stem contained a full helical turn, a true knot would be formed⁴. We present evidence from single-strand specific (S₁) and double-strand specific (V₁) nuclease digestion, that a short RNA oligonucleotide (19 nucleotides long) adopts a stable pseudoknotted structure. The nuclease digestion and thermodynamic properties of this oligonucleotide were compared with those of oligonucleotides which form hairpin structures containing the two possible stem regions in the pseudoknot. These results show that appropriate sequences can form pseudoknots and indicate that pseudoknots are a significant type of local tertiary structure which must be considered in the folding of complex RNA molecules.

Pseudoknotting and true knotting of RNA are not considered by present algorithms used to predict secondary structure^{4,5}; knots violate the basic premise that the free energies of separate regions are additive. Previous experimental evidence for

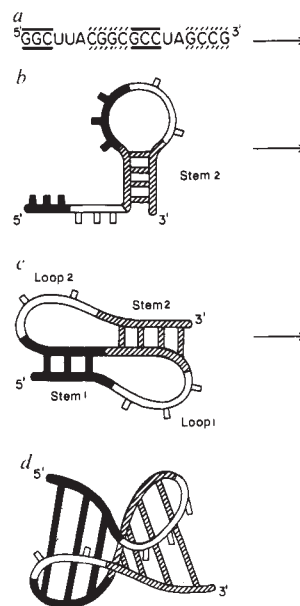


Fig. 1 A schematic representation of a possible folding of PK1 into a pseudoknot. *a*, The primary sequence of PK1; the nucleotides denoted with a bold line correspond to the shaded regions of the schematic drawings (stem 1) and those which are hatched correspond to the hatched regions (stem 2). *b*, A four bp stem (stem 2) formed at the 3' end of the molecule. *c*, A pseudoknot occurs when three bases at the 5' end pair with bases within the original loop to form an additional stem (stem 1) and loop region (loop 1). *d*, A drawing of the postulated three-dimensional folding of the oligonucleotide shows coaxial stacking of the two stem regions to form a seven bp continuous helix. The order for folding the stems has been arbitrarily chosen. The loop regions are nonequivalent; loop 1 bridges the major groove across the four base-pair stem, whereas loop 2 crosses the three bp stem over the minor groove⁸.

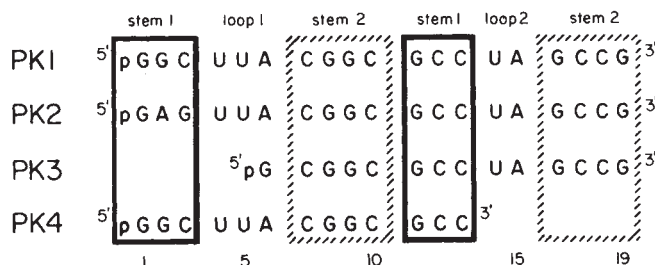


Fig. 2 The primary sequences of the RNA oligonucleotides PK1, PK2, PK3 and PK4. The stem and loop regions are indicated. The numbering of the nucleotides is based on PK1.

Methods. The RNA oligonucleotides were synthesized using T7 RNA polymerase and a synthetic DNA template with a double-stranded promoter region and a long 5' overhang corresponding to the template region²⁷. The polymerase was purified as described by Davanloo *et al.*²⁸. Transcription reactions were incubated at 37 °C for 90 min in 40 mM Tris (pH 8.1 at 37 °C), 1 mM spermidine, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100, 80 mg per ml polyethylene glycol (relative molecular mass (M_r) = 8,000), 31.5 mM $MgCl_2$, 4 mM each nucleotide triphosphate and 5 mM GMP. The DNA concentration was 0.4 μ M per strand and T7 RNA polymerase was used at 30 units per μ L. After addition of EDTA to 50 mM, reaction mixtures were extracted with phenol and precipitated with ethanol. The full length transcript was isolated from a 20% denaturing polyacrylamide gel. The sequence of each oligonucleotide was verified by subjecting internally labelled transcripts to complete enzymatic hydrolysis and identifying the smaller products by two-dimensional TLC²⁹. The same method was used to insure that priming with monophosphate was >95% efficient. A BioRad TSK-125 gel-filtration column was used to assure that PK1-PK4 were monomeric in 0.35 mM $MgCl_2$, 10 mM sodium phosphate, pH 7.0. The retention times of these molecules were compared with the retention times of RNA molecules of known structure and size.

pseudoknotted RNA has been obtained from enzymatic digestion, chemical mapping and sequence comparison of complicated systems including viral RNAs of plants⁹⁻¹⁸ and ribosomal RNAs. To simplify physical studies on this type of structure, we have synthesized a 19-base RNA oligonucleotide (PK1) designed to form a pseudoknotted structure (Fig. 1). Three RNA oligonucleotides corresponding to structural elements of PK1 were also synthesized to use as controls. Oligonucleotide PK2 is 19 bases long, but the 5' end lacks complementary bases necessary to form one of the stems of the pseudoknot; PK3 and PK4 are fragments corresponding to each of the stem and loop pairs possible in the pseudoknot (Fig. 2). Single-strand- and double-strand-specific nucleases were used to probe the structures of the molecules. Thermal melting of the oligonucleotides measured by ultraviolet absorption allowed us to compare the thermodynamic stability of the pseudoknot to that of each individual hairpin. Each molecule was shown to be more than 99% monomeric by gel-filtration chromatography under the RNA and buffer conditions used in both enzymatic digestions and melting experiments.

Structure-mapping experiments using nuclease digestion were performed under conditions specified in the legend for Fig. 3. At 37 °C the single-strand-specific nuclease S_1 cuts PK1 most strongly after the first nucleotide (G_1), probably due to fraying at the end of the helix; this cleavage is seen in double-stranded control oligonucleotides including $r(CGCGCG)_2$. Weaker cutting occurs after U_4 , U_5 and A_6 in loop 1, U_{14} and A_{15} in loop 2, and C_{13} . S_1 does not cut after C_7 , G_8 , G_9 or C_{10} in PK1, evidence for formation of stem 2 (hatched areas of Fig. 3). These positions would be cleaved, as they are in PK4, if the molecule existed as an equilibrium mixture of molecules containing only stem 1 (5' hairpin) or stem 2 (3' hairpin). The links after G_2 and C_3 of PK1 are not accessible to S_1 , consistent with a double-stranded region at the 5' end of the molecule (stem 1, shaded areas of

Fig. 3). In contrast, S_1 cleavage of PK2, which cannot form stem 1, does occur after each of the first three residues (although only weakly after G_3). Thus, stem 1 and stem 2 regions of PK1 are both inaccessible to S_1 cutting; this indicates a pseudoknot.

Almost every phosphodiester link in PK1 is susceptible to hydrolysis by the double-strand-specific nuclease V_1 . Comparison of the V_1 -digestion pattern of PK1 with those of the three hairpins provides evidence for the formation of stem 1 of the pseudoknot. Cleavage after G_2 and C_3 at the 5' end of PK1 is as observed in PK4 which forms this three-base stem. Almost no cutting occurs at the 5' end of PK2 which has a single-stranded 5' end. Cleavage is also observed in PK1 after residues G_{11} and C_{13} in the 3' half of stem 1. The similarity of the digestion pattern at the 3' end of PK1 with those of PK2 and PK3 and the intense cleavage after residues C_7 , G_8 , G_9 and C_{10} of PK1 are consistent with formation of stem 2 of the pseudoknot.

Previous studies²⁰⁻²² on the specificity of V_1 suggest that it recognizes a phosphate backbone characteristic of a stacked base, whether or not the base is involved in a Watson-Crick base pair. Considerable experimental data on both DNA^{23,24} and RNA²⁵ hairpins show that the loop residues are at least partially stacked. V_1 -digestion data presented here on the hairpins PK3 and PK4 are consistent with stacking of loop residues on the 3' side of the hairpin stem. Cleavage by S_1 as well as V_1 shows that these are single-stranded residues. Stacking in loop regions in PK1 explains cutting by V_1 after positions U_4 , A_6 and U_{14} (which are also cleaved by S_1). The link after A_{15} is not cut at all and the U_5 - A_6 link is cut only weakly. These residues bridge the two stems in the pseudoknot. Digestions of the hairpins PK3 and PK4 indicate that the cutting of a stem by V_1 is asymmetric, consistent with recognition and cleavage of one strand at a time²⁰. This asymmetry is apparent in both stem regions of PK1. Absence of cuts after the first nucleotide at the 5' end of stems is consistent with the proposed binding site of V_1 at the 5' end of a duplex in which the enzyme always cleaves at least one nucleotide ahead towards the 3' end²⁰.

Enzymatic mapping provides evidence that the structure formed by PK1 is a pseudoknot. Digestion with the double-strand-specific nuclease V_1 shows that the two stem regions form. Specifically, V_1 cleaves after G_2 , C_3 , G_{11} and C_{13} in the proposed stem 1. Strong cutting in the C_7 - C_{10} region and the similarity of the digestion pattern at the 3' end of PK1 with that of PK2 and PK3 show that stem 2 does form. Single-strand cleavage by S_1 occurs only in the proposed loop regions and not elsewhere in the molecule; for example, no cleavage occurs at the 5' end of PK1, consistent with formation of both stem regions within the same molecule.

The thermal stability of the structures formed by oligonucleotides PK1 and PK2 was probed by digestion with S_1 at 37 °C, 60 °C and 80 °C (Fig. 3). At 60 °C the digestion patterns for both molecules remain essentially unchanged from those at 37 °C. Only at 80 °C are all sites on the molecules accessible to the enzyme. The results of digestion by S_1 at various temperatures suggest that the structures formed by both PK1 and PK2 are stable to at least 60 °C. At 37 °C PK1 is more resistant to cleavage by S_1 than any of the control molecules.

Thermal denaturation of the RNA molecules was monitored using ultraviolet (UV) absorbance. The thermodynamic properties of PK1 were compared to those of the three control hairpins. The melting curves for PK1, PK3 and PK4 are shown in Fig. 4a. Melting temperatures (T_m), ΔH° , ΔS° and ΔG° (at 37 °C) (standard enthalpies, entropies and free energies, respectively) are summarized in Fig. 4b. The pseudoknot, PK1, melts as a single transition with a T_m of 73 °C, intermediate between the melting temperatures of the 3' hairpin, PK3 (T_m = 78 °C), and the 5' hairpin, PK4 (T_m = 64 °C). The T_m s of these transitions are independent of strand concentration over a 20-fold range. PK1 appears to melt in a two-state, all-or-none transition. The thermodynamic stability, as well as the enzymatic digestion, suggests that this structure is neither of the two possible hairpins nor an

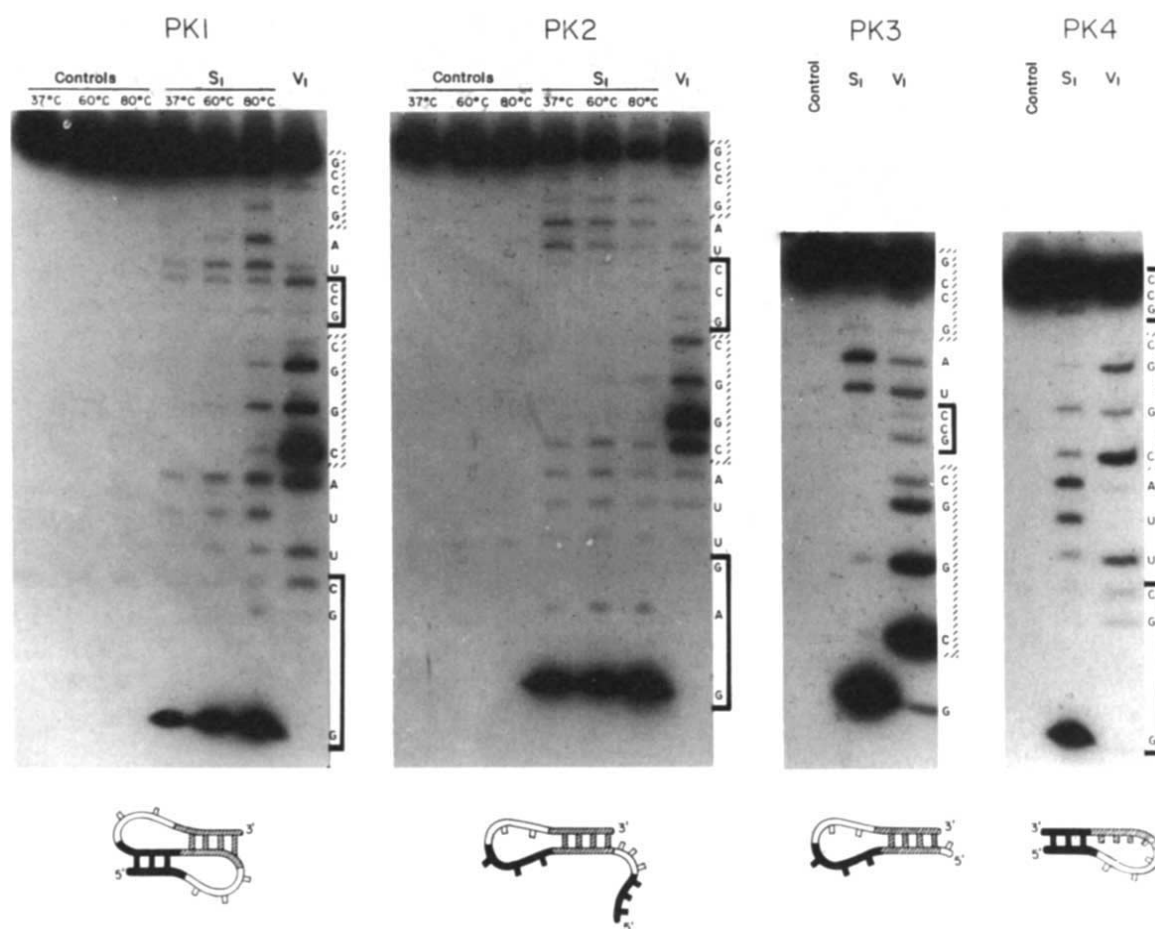


Fig. 3 Autoradiographs of gel-electrophoretic analysis of S₁ and V₁ digestions of PK1, PK2, PK3 and PK4. The sequence of each molecule is written to the right of the V₁-digestion lane. Below each autoradiograph is a schematic diagram of the folded molecule. The stem 1 regions are shaded and the regions corresponding to stem 2 are hatched. Bands which appear in control lanes are due to non-specific hydrolysis, which leaves a 3' phosphate. These fragments have migrated further than those of comparable length generated by digestion with either S₁ or V₁, which both leave a 3'-hydroxyl group.

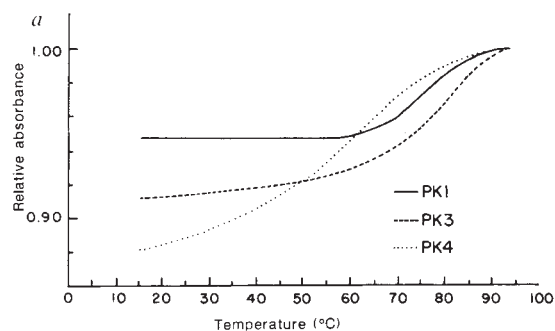
Methods. RNA used in enzymatic-digestion experiments was dephosphorylated, 5'-end labelled with ³²P and purified from a 20% polyacrylamide denaturing gel. Each enzymatic digestion reaction contained about 1 pmol of labelled RNA and 3 µg unfractionated yeast tRNA in 20 µl. Each reaction was heated to 95 °C for 1 min in the appropriate buffer, cooled on ice, then incubated for 1 min at the appropriate temperature before addition of the enzyme. Digestions with V₁ were carried out at 37 °C for 2 min in 10 mM NaCl, 0.35 mM MgCl₂, 5 mM Tris (pH 7.0 at 37 °C) using 0.35 units of V₁ (Pharmacia). The digestion pattern of V₁ is the same at 10-fold lower enzyme concentration. Digestions with S₁ were carried out in 0.35 mM MgCl₂, 10 mM NaCl, 5 mM 2-(*N*-morpholino)ethansulphonic acid (pH 6.3 at the specified temperature) using 12 units of S₁ (Pharmacia). Digestions with S₁ at 37 °C, 60 °C and 80 °C of PK1 and PK2 were incubated for 3 min, 1 min and 2 min, respectively, to account for the temperature dependence of the enzymatic activity. Digestions of PK3 and PK4 were carried out at 37 °C for 3 min. The digestion pattern of S₁ is unchanged at pH 7.0, but a pH of 6.3 was used to allow shorter reaction times. Cutting patterns for S₁ and V₁ were the same in the presence and absence of tRNA. Reactions were stopped by addition of 1 volume of 9 M urea in 50 mM Tris, 50 mM boric acid, 1 mM EDTA buffer and freezing at -70 °C. Control reactions without enzyme were treated exactly as digestions using S₁ buffer conditions. Reactions were analysed using 20% denaturing polyacrylamide gels and autoradiography. Autoradiographs shown were developed after 12 h.

equilibrium mixture between the two. The experimental free-energy difference between the 3' hairpin and the 5' hairpin is ~3 kcal mol⁻¹ at 37 °C, favouring the formation of the 3' hairpin by a factor of 130. If the free energies of the 3'- and 5'-hairpin structures for PK1 can be approximated by the free energies of PK3 and PK4, the concentration of the 5' hairpin should be negligible (<1%) at 37 °C. There is little difference in free energy between the pseudoknot, PK1, and the 3' hairpin, PK2 or PK3, at 37 °C; this suggests that PK1 may exist in equilibrium between a pseudoknotted and a 3'-hairpin structure.

The major contributions to the enthalpy of the transitions are the stacking interactions between neighbouring aromatic bases. The ΔH° of melting PK1 is 55 kcal mol⁻¹ which exceeds the ΔH° of melting both PK3 (46 kcal mol⁻¹) and PK4 (34 kcal mol⁻¹); the enthalpies are assumed to be independent of temperature. The formation of an additional stem and loop region

in a pseudoknot would explain a higher ΔH° for PK1. Yet this 9 kcal mol⁻¹ stabilization is not as great as that predicted⁷ by the formation of three stacked base pairs. We postulate that three additional stacking interactions are not gained in forming the 5' stem as the loop residues are already stacked in the single-hairpin structures. The pseudoknot PK1 thus gains little enthalpic stability over the 3' hairpin formed by PK2 and PK3. The hypochromicity of melting of PK1 (5%) which is lower than that of PK3 (8%) or PK4 (10%) could be a result of perturbed stacking within the stem regions of PK1.

Modelling²⁶ of the pseudoknotted structure at the 3' end of turnip yellow mosaic virus RNA shows that the two stem regions can coaxially stack to form a continuous A-like helix which mimics the acceptor stem of tRNA. Model building of PK1 in our laboratory shows that whereas the connecting loop of three nucleotides should easily bridge stem 1, bridging stem 2 with



b	Molecule	T_m (°C)	ΔG (at 37°C) (kcal mol ⁻¹)	ΔS (at 37°C) (cal mol ⁻¹ K ⁻¹)	ΔH (at 37°C) (kcal mol ⁻¹)
	PK1	73	-5.4	-160	-55
	PK2	77	-5.3	-154	-53
	PK3	78	-5.7	-130	-46
	PK4	64	-2.4	-102	-34

Fig. 4 a, Normalized UV-absorbance melting curves for PK1, PK3 and PK4 in 0.35 mM MgCl₂, 10 mM sodium phosphate, pH 7.0. Data were collected at 260 nm and smoothed. RNA strand concentrations were 2 μ M. From multiple acquisitions of data, we estimate a precision of ± 0.5 absorbance units in the hypochromicity. The shape and T_m values of the melting curves for PK2, PK3 and PK4 are unchanged over a strand concentration range from 1 μ M to 20 μ M. At concentrations of PK1 strands between 2.5 μ M and 10 μ M the lower baseline of the melting curve has a positive slope, yet the T_m of the transition is unchanged. The shape of the melting curve for PK2 (not shown) is similar to that of PK3, except that the former has a steeper sloping lower baseline. This sloping baseline introduces a greater error in the determination of thermodynamic parameters for PK2 than for the other molecules (see below). **b**, Table of thermodynamic parameters for PK1, PK2, PK3 and PK4 in 0.35 mM MgCl₂, 10 mM sodium phosphate, pH 7.0. The melting temperature (T_m) is defined as the midpoint of the transition. Values of ΔG° are given at 37°C. Estimated precisions in the various quantities are: $T_m \pm 1^\circ\text{C}$, $\Delta G^\circ \pm 0.2$ kcal mol⁻¹, $\Delta S^\circ \pm 10$ cal mol⁻¹ K⁻¹, $\Delta H^\circ \pm 5$ kcal mol⁻¹ (except for PK2 where the corresponding precisions are, $\Delta H^\circ \pm 10$ kcal mol⁻¹, $\Delta S^\circ \pm 15$ cal mol⁻¹ K⁻¹, $\Delta G^\circ \pm 0.3$ kcal mol⁻¹ due to uncertainty in the choice of baseline).

Methods. UV-absorbance melting curves were obtained on a Gilford Model 250 UV-vis spectrophotometer with a Gilford Model 2527 thermoprogrammer. Melting curves were analysed assuming a two-state transition. Fraction versus temperature and equilibrium constants were calculated as previously described³⁰. Enthalpies were calculated using van't Hoff analysis of the entire fraction versus temperature profile and compared to the ΔH° obtained using the slope³⁰ of the curve of fraction versus temperature at the T_m . Values obtained by the two methods differed by less than 0.5 kcal mol⁻¹. Values of ΔS° were calculated using the relationship $\Delta S^\circ = \Delta H^\circ / T_m$. Melting curves were obtained over an RNA concentration range of 1 μ M to 20 μ M.

two nucleotides can result in a distortion of stem or loop structure. A distortion would be consistent with the low hypochromicity and enthalpic stabilization of PK1 in comparison to PK3. In general, the equilibrium between pseudoknotted and hairpin structures will depend on the relative free-energy contributions of the stem and loop regions in each structure. A systematic study of a variety of sequences is needed to determine these free-energy contributions, especially that of the loop regions in the pseudoknot. Elucidation of the conformation of a pseudoknot will require more detailed spectroscopic studies including NMR. The results detailed here indicate that pseudoknots are significant structural motifs which should be considered in prediction of the tertiary interactions in complex RNA molecules. Pseudoknots may be especially important as a means of bringing

two separated nucleotides in a single strand into close spatial proximity.

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Errata

The origin of the clockwork-escapement

Nature **330**, 615 (1987).

THE name and address for the author of this piece of Scientific Correspondence were given incorrectly in the 17 December issue, and should read: Annie Lantink-Ferguson, B. de Beaufortweg 61, 3833 AE Leusden-Centrum, The Netherlands.

Limits on quantitative information from high-resolution electron microscopy of YBa₂Cu₃O₇ superconductors

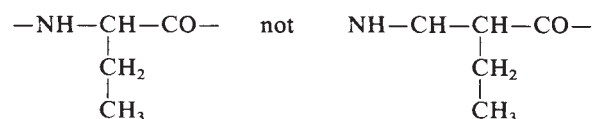
N. P. Huxford, D. J. Eaglesham & C. J. Humphreys
Nature **329**, 812-813 (1987).

IN this letter the images for Figs 1 and 2 were transposed.

No requirement of cyclic conformation of antagonists in binding to vasopressin receptors

M. Manning, J. P. Przybylski, A. Olma, W. A. Klis, M. Kruszynski, N. C. Wo, G. H. Pelton & W. H. Sawyer
Nature **329**, 839-840 (1987).

IN this letter, the structure for α -aminobutyryl in the footnotes to Table 1 is incorrect. It should be:



In addition, the page numbers in ref. 1 should read 802-870, not 802-807.